T-SPOT.

PACKAGE INSERT

For *In Vitro* Diagnostic Use Only

This package insert covers use of both:
T-SPOT.*TB 8* (Multi-use 8-Well Strip Plate Format. Catalogue number: TB.300)
T-SPOT.*TB 96* (Single-use 96-well Plate Format. Catalogue number: TB.200)

Caution: Federal (USA) law restricts this device to sale by or on the order of a licensed health care professional

Oxford Immunotec

Harnessing the power of T cell measurement

PI-TB-US-V1

INDEX

1. INTENDED USE

T-SPOT®.*TB* is an in vitro diagnostic test for the detection of effector T cells that respond to stimulation by *Mycobacterium tuberculosis* antigens ESAT-6 and CFP-10 by capturing interferon gamma (IFN-γ) in the vicinity of T cells in human whole blood collected in sodium citrate or sodium or lithium heparin. It is intended for use as an aid in the diagnosis of *M. tuberculosis* infection.

T-SPOT.*TB* is an indirect test for *M. tuberculosis* infection (including disease) and is intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations.

2. SUMMARY & EXPLANATION

It is estimated that 15 million Americans are infected with *M. tuberculosis*[1]. Each person carrying latent TB infection (LTBI) has approximately a 10% chance of progression to active TB disease[1]. This risk is elevated among certain groups, including those who have been recently infected and those who have clinical conditions that are associated with an increased risk for progression of LTBI to active TB.

Historically, TB infection screening was performed with the Tuberculin Skin Test (TST). The recommended technique for administering the TST in the US is the Mantoux method[2]. The Mantoux skin test involves the intracutaneous injection of tuberculin (also called Purified Protein Derivative – or PPD) into the volar surface of the forearm. TST results are read 48-72 hours after administration; this interval being required for the development of a Delayed-Type Hypersensitivity (DTH) reaction. The extent of induration at the site of injection is measured and recorded in mm. Three different cutoffs for the TST are used to increase its specificity; based on the characteristics of the patient being tested.

In addition to the TST, there are ELISA-based assays for detecting IFN-gamma secretion from lymphocytes stimulated with TB-specific antigens[3]. This method uses a principle similar to that of T-SPOT.*TB*, by measuring the amount of IFN-gamma in blood using the ELISA technique.

T-SPOT.*TB* is an in vitro diagnostic test based on an enzyme-linked immunospot (ELISPOT) method. The test enumerates effector T cells responding to stimulation with a combination of peptides simulating ESAT-6 and CFP10 antigens. ESAT-6 and CFP10 are absent from all BCG strains and from most non-tuberculous mycobacteria with the exception of *M. kansasii, M. szulgai* and *M. marinum*[4-6]. In contrast, individuals infected with *M. tuberculosis* complex organisms (*M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti*) usually have T cells in their blood which recognize these and other mycobacterial antigens.

T-SPOT.*TB* can be used in screening of risk groups for TB infection. In addition, T-SPOT.*TB* can be used as a diagnostic aid when evaluating patients suspected of having active TB disease.

The T-SPOT.*TB* test has been tested in some patient groups indicated for screening for TB infection according to current ATS/CDC Guidance[2]: such as, human immunodeficiency virus (HIV) positive persons, recent contacts of TB case patients, residents and employees of high-risk congregate settings, chronic renal failure, children younger than 4 yr of age or infants, children, and adolescents exposed to adults at high-risk and immunosuppressed patients

Refer to the most recent CDC guidance (http://www.cdc.gov/nchstp/tb) for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

PRINCIPLE OF TEST

The immune response to infection with *Mycobacterium tuberculosis* is mediated predominantly through T cell activation. As part of this response, T cells are sensitized to *M. tuberculosis* antigens and the activated effector T cells, both CD4+ and CD8+, produce the cytokine interferon gamma (IFN-γ) when stimulated by these antigens[7-8]. T-SPOT.*TB* uses the enzyme-linked immunospot (ELISPOT) methodology to enumerate *M. tuberculosis*-sensitized T cells by capturing interferon-gamma (IFN-γ) in the vicinity of T cells from which it was secreted.[9]

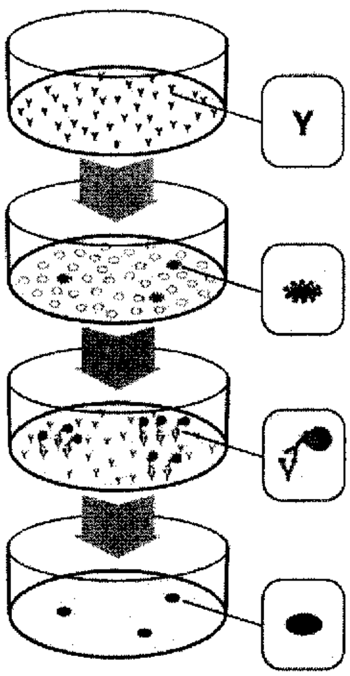
Peripheral blood mononuclear cells (PBMCs) are separated from a whole blood sample, washed and then counted before being added into the assay.

Isolated PBMCs (white blood cells) are placed into microtiter wells where they are exposed to a phytohemagglutinin (PHA) control (a mitogenic stimulator indicating cell functionality), nil control, and two separate panels of *M. tuberculosis* specific antigens. The PBMCs are incubated with the antigens to allow stimulation of any sensitized T cells present.

Secreted cytokine is captured by specific antibodies on the surface of the membrane, which forms the base of the well, and the cells and other unwanted materials are removed by washing. A second antibody, conjugated to alkaline phosphatase and directed to a different epitope on the cytokine molecule, is added and binds to the cytokine captured on the membrane surface. Any unbound conjugate is removed by washing. A soluble substrate is added to each well; this is cleaved by bound enzyme to form a (dark blue) spot of insoluble precipitate at the site of the reaction.

Evaluating the number of spots obtained provides a measurement of the abundance of *M. tuberculosis* sensitive effector T cells in the peripheral blood. These principles behind the T-SPOT assay system are described in Figure 1 below.

Figure 1: Principles of the T-SPOT assay system. For illustration only, refer to Section 6, Instructions for Use for detailed procedural instructions.



First a blood sample is collected from the patient. The white blood cells are separated and then put into the wells of a 96-well microtiter plate. The plates are pre-coated with high affinity antibodies (Y) to Interferon-gamma (IFN-γ), a cytokine released by effector T-cells when fighting TB infection.

Antigens from the TB organism are then added to the wells with the white blood cells to provoke IFN-γ secretion from any effector T cells primed against TB. The antigens are selected to be unique for *M. tuberculosis* so that only responses of T cells specific for TB are measured.

Antigen specific responding T cells ( ) release the cytokine (•), which is captured in the immediate vicinity of the T cells, by the antibodies lining the bottom of the well.

After a short incubation time the wells are washed, removing the antigens and cells from the wells. A conjugated second antibody ( ) is then added which binds to the IFN-γ secreted by the T cells (and captured by the primary antibody).

A subtrate is then added which produces spots ( ) where the IFN-γ was secreted by T cells. The number of spots is counted.

34

3. REAGENTS & STORAGE

MATERIALS PROVIDED

T-SPOT.*TB 8* (Multi-use 12 x 8-well strip version) contains:

1. 1 microtiter plate: 96 wells, supplied as 12x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ).
2. 2 vials (0.8mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.8mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.8mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50μL) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT^{plus} solution.
7. CD containing the package insert

T-SPOT.*TB 96* (Single-use solid 96-well plate version) contains:

1. 1 microtiter plate: 96 wells coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ).
2. 2 vials (0.7mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.7mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.7mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50μL) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT^{plus} solution.
7. CD containing the package insert

STORAGE & STABILITY

Store the unopened kit at 2-8°C. The components of the kit are stable up to the expiration date printed on the kit box, when stored and handled under the recommended conditions. The kit must not be used beyond the expiration date on the kit label.

For T-SPOT.*TB 8,* store opened kit components at 2-8°C. Opened components must be used within 8 weeks of opening, such period ending no later than the expiration date on the kit label. **Avoid prolonged exposure of the Substrate Solution to light.**

T-SPOT.*TB 96* is a single-use kit and, once opened, all materials should be used immediately and not reused. Do not mix components between different kits.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

1. 8-well strip plate frame (available from Oxford Immunotec).
2. BLII cabinet (recommended).
3. Blood collection tubes, such as Vacutainer® CPT™ or heparinized tubes (Note: CPT tubes are available from Oxford Immunotec).
4. Ficoll (if not using CPT tubes)
5. A centrifuge for preparation of PBMCs (capable of at least 1800 RCF (g) and able to maintain the samples at room temperature (18-25°C)) if using density centrifugation methods to separate the PBMCs.

6. Equipment and reagents to enable counting of PBMCs; either manually using Trypan Blue (or other appropriate stain) and a hemocytometer on a microscope or automatically using a suitable hematology analyzer.
7. A humidified incubator capable of 37 ± 1°C with a 5% CO_2 supply.
8. An automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates.
9. Adjustable pipettes to cover a range of volumes from 1-1000µl (such as four Gilson pipettes capable of delivering volumes of 1-10µL, 2-20µL, 20-200µL and 100-1000µL) and sterile pipette tips.
10. Sterile PBS solution: such as GIBCO™ 1x D-PBS (Invitrogen; catalogue number 14040-133).
11. Distilled or deionized water.
12. A means of visualizing the wells, or capturing a digital image of the well, such as a stereomicroscope, magnifying glass or plate imager to allow counting of spots.
13. Sterile cell culture medium such as GIBCO AIM-V™ (Invitrogen; catalogue number 31035-025 research grade). (Note: AIM-V media is available from Oxford Immunotec). **The use of this serum free medium for the incubation step is strongly recommended.** RPMI 1640 (Invitrogen; catalogue number 11875-093) may be used in the initial sample preparation steps only. It is recommended that cell culture media are stored in appropriate aliquots and excess material is discarded after use. **Cell culture media should be pre-warmed to 37°C before use with T-SPOT.*TB*.** To avoid problems with contaminated media, it can be helpful to dispense bottles of AIM-V or RPMI 1640 into smaller aliquots.

4. WARNINGS & PRECAUTIONS

- For *in vitro* diagnostic use only
- Operators should be trained in the assay procedure and be sure they understand the instructions for use before performing the assay.
- Read the assay instructions carefully before use. Deviations from the instructions for use in this package insert may yield erroneous results.
- Care should be taken when handling material of human origin. All blood samples should be considered potentially infectious. Handling of blood samples and assay components, their use, storage and disposal should be in accordance with procedures defined in appropriate national, state or local biohazard and safety guidelines or regulations.
- Care should be taken when working with chemicals. All chemicals should be considered potentially hazardous. A material safety data sheet for the kit is available from Oxford Immunotec.
- Discard unused reagents and biological samples in accordance with Local, State and Federal regulations.
- The correct number of cells must be added to each well. Failure to do so may lead to an incorrect interpretation of the result.
- Do not mix components from different kit lots.
- Observe aseptic technique to avoid contaminating the reagents, assay wells, cell suspensions and cell culture media.
- Variation to the stated pipetting and washing techniques, incubation times and/or temperatures may influence the actual results obtained and should be avoided.
- Blood should be collected and processed as soon as possible.
- Store and transport blood samples to the laboratory at room temperature (18-25°C). Do not refrigerate or freeze whole blood samples.
- Failure to adhere to the recommended incubation times and temperatures may lead to an incorrect interpretation of the results.
- Indentations in the membrane caused by pipette or well washer tips may develop as artifacts in the wells which could confuse spot counting.

5. SPECIMEN COLLECTION & HANDLING

Individual laboratories should validate their procedures for collection and separation of PBMCs to obtain sufficient numbers. It is recommended that:

- Blood samples are collected into either sodium citrate or sodium heparin Vacutainer® CPT tubes (Becton Dickinson) and then the PBMCs separated in the tube using the manufacturer's instructions.
- Blood samples are collected into lithium heparin blood collection tubes with PBMCs being subsequently separated using standard separation techniques such as Ficoll-Paque®. Alternative methods to purify the PBMC fraction may be employed if desired. **Note EDTA tubes are not recommended.**
- A patient's cells can be pooled, if necessary to obtain sufficient cells from multiple tubes of blood which were collected and processed concurrently.

Typically, for an immunocompetent patient, sufficient PBMCs to run the assay can be obtained from venous blood samples according to the following guidelines:

- Adults and children 10 years old and over: one 8mL or two 4mL tubes (CPT) or two lithium heparin 6mL tubes
- Children 2-9 years old: one 4mL tube (both methods)
- Children up to 2 years old: one 2mL pediatric tube (both methods)

Blood samples should be processed within 8 hours. Samples may be successfully stored for longer periods of time but users should validate this in their own setting[9-10]. **Whole blood samples should be maintained between 18°C and 25°C until processed**. The instructions below give more information on specimen collection steps:

1. Collect a blood sample according to the instructions supplied with the collection device. The tube contents must be inverted (8 – 10 times) to ensure that the whole blood is mixed thoroughly with the anticoagulant. Store collected blood at room temperature (18-25°C). **Do not refrigerate or freeze.**
2. For CPT blood collection tubes, centrifuge 8mL CPT tubes at 1600 RCF(g) for 28 minutes or 4mL CPT tubes at 1800 RCF (g) for 30 minutes at room temperature (18-25°C). If using Ficoll-Paque™ Plus, dilute the blood with an equal volume of RPMI 1640 medium (1 part blood to 1 part RPMI). Layer carefully the diluted blood onto Ficoll-Paque Plus (2-3 parts diluted blood to 1 part Ficoll-Paque) and centrifuge at 1000 RCF (g) for 22 minutes at room temperature (18-25°C).

   Note: Review the manufacturer's instructions before using either CPT tubes or Ficoll-Paque. Ensure the tubes are centrifuged at the correct speed. The speeds given above are in g or Relative Centrifugal Force (RCF). This is not the same as Revolutions Per Minute (RPM). If the centrifuge will only measure speed in RPM, then convert to the recommended RCF value by measuring the rotor radius and using a conversion table. Leucosep tubes (Greiner Bio-One) offer a time-saving approach to Ficoll gradients. The tubes contain a porous barrier that enables the blood sample to be poured onto the Ficoll gradient, thereby eliminating the need to gently layer on the sample.
3. Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Bring the volume to 10mL with cell culture medium. **Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs.**

   Circulating factors in whole blood samples are known to interfere with whole blood interferon gamma tests, e.g., rheumatoid factor, heterophilic antibodies, and pre-existing amounts of interferon gamma. The separation and washing of the PBMCs enables removal of these potentially interfering substances prior to performing the assay.

   Note: After centrifugation, PBMCs should be extracted using a large bore (e.g. 1mL) pipette tip, by immersing the pipette tip into the PBMC layer. This cloudy layer should be carefully aspirated and transferred to a sterile conical tube for the wash steps. Ensure that all of the cloudy PBMC layer is collected. It is better to take more of the plasma layer than to leave any of the PBMCs in the blood collection tube. However, if using CPTs avoid transferring any of the separation gel, which can block the tip. If this happens transfer the cells already in the tip into a centrifuge tube and then use a new tip

37

to transfer the remaining PBMCs. A variety of media can be used for washing the cells during these steps 3-5; both AIM-V and RPMI 1640 have been used successfully and are recommended.

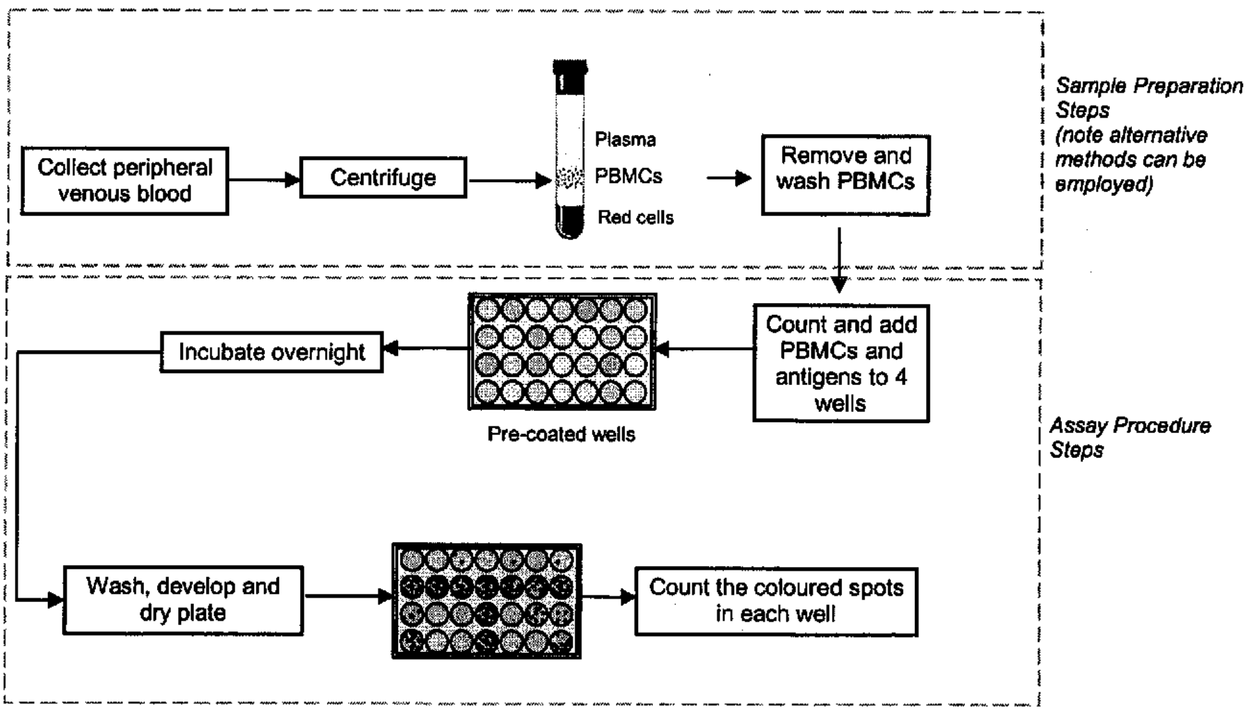
4. Centrifuge at 600 RCF (g) for 7 minutes. Pour off the supernatant and resuspend the pellet in 1mL medium.
5. Bring the volume to 10mL with fresh medium and centrifuge at 350 RCF (g) for 7 minutes.
6. Pour off the supernatant and resuspend the pellet in 0.7mL cell culture medium. **The serum-free medium AIM-V has been used successfully and is strongly recommended**

   Note: *Steps 2-6 should be performed in a BLII cabinet to protect the user and prevent contamination of samples.*

6. INSTRUCTIONS FOR USE

A full T-SPOT.*TB* plate will process 24 patient samples. The performance of the assay is typically carried out on the afternoon of one day and the morning of the following day, to allow the 16-20hr incubation step to take place overnight. If this timing is used, then on the afternoon of day 1 the blood samples are processed to prepare the PBMCs for the assay and the assay is initiated by adding the PBMCs and antigens to the assay plate and placing the plate into the incubator. On day 2, the plate is removed from the incubator and the development steps are performed and the plate is read. The time to process a full plate is approximately 3 hours of elapsed time (actual hands-on labor time will be less due to the centrifugation steps) on day 1 and 30 minutes of labor time (not including the 1 hour incubation of the secondary antibody and time for plate drying) on day 2. The procedure for conducting the test is summarized in Figure 2 and further described below:

Figure 2: Diagram illustrating the main steps required to perform T-SPOT.TB. Note that not all 96 wells of the plates are shown in the illustration.



38

REAGENT PREPARATION

1. The vials of *M. tuberculosis* ESAT-6 antigens (Panel A), *M. tuberculosis* CFP10 antigens (Panel B) and the Positive Control are supplied ready to use.
2. Prepare a 1:200 dilution working Conjugate Reagent solution. Calculate the volume of working Conjugate Reagent solution required. Conjugate Reagent can be made to working strength and stored at 2-8°C up to six weeks before using in the assay.

   Note: Each patient sample uses 4 wells. 50µL diluted Conjugate Reagent will be added to each well. Thus, for one strip (2 samples, 8 wells), prepare 500µL of working strength solution by adding 2.5µL of concentrated Conjugate Reagent to 497.5µL PBS. For one 96-well plate (24 samples), prepare 5mL of working strength solution by adding 25µL of concentrated Conjugate Reagent to 4975µL PBS.
3. The Substrate Solution is supplied ready to use. Prior to removing the plate from the incubator (day 2) remove the substrate solution from storage and allow to equilibrate to room temperature.

CELL COUNTING AND DILUTION

T-SPOT.*TB* requires 250,000± 50,000 PBMCs per well. A total of four wells are required for each patient sample; thus 1 X 10^6 PBMCs are required per patient. The number of *M. tuberculosis* T cells in the specimen is normalized to a fixed number of PBMCs.

1. Perform a PBMC count. Cells can be counted by a variety of methods, including manual counting using Trypan Blue (or other appropriate stain) and a hemocytometer, or using an automated hematology analyzer.
2. Briefly, for manual counting with a Neubauer hemocytometer using Trypan Blue, add 10µL of the final cell suspension to 40µL 0.4%(w/v) Trypan Blue solution. Place an appropriate aliquot onto the hemocytometer and count the cells in the grid. For other types of hemocytometer and for automated devices, follow the manufacturers' instructions.

   Note: Care should be taken to ensure that the cell suspension is well mixed immediately prior to removal of aliquots for counting. Cells can settle towards the bottom of the tube leading to a misinterpretation of the true cell number. Mixing should be done by either gentle swirling of the tube by hand, or by gently agitating the suspension by pipetting the suspension up and down several times.
3. Calculate the concentration of PBMCs present in the stock cell suspension.

   Note: Ensure the calculation is correct for the cell counting system used as the use of either insufficient or excess cells may lead to an incorrect interpretation of the result.
4. Prepare 500µL of the final cell suspension at a concentration of 2.5×10^5 cells/100µL (giving 1.25×10^6 PBMCs in total).

   Note: Ensure cells are thoroughly mixed, by gently agitating the suspension by pipetting the suspension up and down several times, before removing an aliquot for dilution. PBMC numbers between 200,000 and 300,000 per well have been shown to give consistent T-SPOT.TB results.

PLATE SET UP AND INCUBATION

T-SPOT.*TB* requires four wells to be used for each patient sample. A Nil Control and a Positive Control should be run with each individual sample. It is recommended that the samples be arranged vertically on the plate as illustrated below.

Nil Control

Panel A (ESAT-6)

Panel B (CFP10)

Positive Control

Each 96-well plate can process up to 24 patient samples. Use the numbers of plates required for the numbers of samples that you wish to process. The 8-well strip version of T-SPOT.*TB* (T-SPOT.*TB 8*) provides the additional flexibility of 12 x 8-well strips. Each strip will process 2 samples. If you have purchased this version, use only the numbers of strips that you require.

T-SPOT.*TB* is an assay that measures T cell function; no standard curves are required. Therefore each patient will only require 4 wells to be used for each sample. The recommended plate layout for 24 samples is shown below:

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	3N	5N	7N	9N	11N	13N	15N	17N	19N	21N	23N
B	1A	3A	5A	7A	9A	11A	13A	15A	17A	19A	21A	23A
C	1B	3B	5B	7B	9B	11B	13B	15B	17B	19B	21B	23B
D	1M	3M	5M	7M	9M	11M	13M	15M	17M	19M	21M	23M
E	2N	4N	6N	8N	10N	12N	14N	16N	18N	20N	22N	24N
F	2A	4A	6A	8A	10A	12A	14A	16A	18A	20A	22A	24A
G	2B	4B	6B	8B	10B	12B	14B	16B	18B	20B	22B	24B
H	2M	4M	6M	8M	10M	12M	14M	16M	18M	20M	22M	24M

Key: N=nil control, A=Panel A, B=Panel B, M=Mitogen Positive Control

1. Remove the pre-coated 8-well strips from the packaging, clip into a plate frame and allow to equilibrate to room temperature. Remove the required number of strips only, reseal any remaining unused strips and the desiccant pouch in the outer foil packaging and return to storage at 2-8°C. Alternatively, if the 96-well version is being used, remove the plate from the pouch and allow it to equilibrate to room temperature.

   Note: Clip the strips to be used into an empty plate frame fitted with an under cover and lid. Frames, covers and lids should be retained and reused.

2. Add in the Panels and the Controls;

   i. Add 50µL AIM-V cell culture medium to each Nil Control well.
   ii. Add 50µL Panel A solution to each well required.
   iii. Add 50µL Panel B solution to each well required.
   iv. Add 50µL Positive Control solution to each cell functionality control well.

   Do not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause artifacts in the wells.

3. To each of the 4 wells to be used for a patient sample, add 100µL of the patient's final cell suspension (containing 250,000 cells). Use a new tip for the addition of each individual patient's cells to avoid cross-contamination between wells. Take care not to contaminate adjacent wells, by passing liquid from one well to another if pipette tips are reused for multiple wells.

   Note: Ensure mixing (as in the Cell Counting & Dilution steps) before removal of each 100µL aliquot.

4. Incubate the plate with the lid on in a humidified incubator at 37°C with 5% CO2 for 16-20 hours. Avoid disturbing the plate once in the incubator. Do not stack plates as this may lead to uneven temperature distribution and ventilation.

   Note: The CO_2 incubator must be humidified. Check the water dish has sufficient water to ensure a humid atmosphere is achieved.

SPOT DEVELOPMENT AND COUNTING

1. Remove the plate from the incubator and discard the cell culture medium by flicking the contents into an appropriate container.

   Note: At this time remove the Substrate Solution from the kit and allow to equilibrate to room temperature.

2. Add 200µL PBS solution to each well. **Do not use PBS containing Tween™ or other detergents, as this causes high background counts**.

   Note: Use freshly prepared or sterile PBS.

3. Discard the PBS solution. Repeat the well washing an additional 3 times with fresh PBS solution for each wash. An automated washer can be used for the washing steps.

   Note: For washing, a multi-channel pipette or a plate washer may be used. Discard PBS into a suitable container after each wash. Do not use pipettes to remove the PBS as this risks damaging the membrane. If using a plate washer, ensure the manifold is adjusted so that the tips do not touch the membrane. After the final wash, tap the plate on lint-free towel to ensure all PBS is removed – any excess left will further dilute the Conjugate Reagent.

4. If not already prepared during the reagent preparation step; dilute concentrated Conjugate Reagent 200X in PBS to create the working strength solution.

5. Add 50µL working strength Conjugate Reagent solution to each well and incubate at 2-8°C for 1 hour.

   Note: Use of a multi-channel pipette or stepper pipette is recommended. Care should be taken to ensure that the Conjugate Reagent is added to every well as the solution is clear and uncolored - therefore, it may be difficult to see to which wells it has been added.

6. Discard the conjugate and perform the four PBS washes as described in steps 2. and 3. above.

7. Add 50µL Substrate Solution to each well and incubate at room temperature for 7 minutes.

8. Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.

9. Allow the plate to dry by standing the plate in a well ventilated area or in an oven at up to 37°C.

   Note: Spots become more visible as the plate dries; therefore ensure that the plate is thoroughly dry before reading. Allow 4 hours drying time at 37°C or at least 16 hours at room temperature.

10. Count and record the number of distinct, dark blue spots on the membrane of each well. Apply the Results Interpretation and Assay Criteria (see below) to determine whether a patient sample is 'Positive' or 'Negative'. **The spots produced as a result of antigen-stimulation should appear as large, round and dark spots. Often a gradient effect can be observed with a darker centre and a more diffuse periphery. Non specific artifacts that can occur are smaller, less intense and irregular in shape.**

    Note: Spots can be counted directly from the well using a magnifying glass or stereomicroscope or from a digital image captured from a microscope.

Once developed, the completed assay plates remain stable and they do not therefore need to be read immediately. The plates may be archived for retrospective quality control or re-examination for up to 12 months if kept in a dry, dark environment at room temperature.

QUALITY CONTROL

A typical result would be expected to have few or no spots in the Nil Control and 20 or more spots in the Positive Control (see Figures 4a & b for typical results from the US clinical study).

High numbers of spots in the Nil Control may occur. In addition, high background staining in one or more wells may occur which makes counting of spots difficult. If high background staining occurs such that discrimination of the spots from the background is hindered, the results should be considered invalid. These results are usually due to operator issues, such as suboptimal plate washing, medium contamination or inappropriate specimen handling and PBMC separation methods. It is, however, possible that the state of health of the patient may produce this effect in a small number of cases.

A Nil Control spot count in excess of 10 spots should be considered as 'Invalid'.

Typically, the cell functionality Positive Control spot count should be ≥20 or show saturation (too many spots to count). A small proportion of patients may have T cells which show only a limited response to PHA[11]. Where the Positive Control spot count is < 20 spots, it should be considered as 'Invalid', unless either Panel A or Panel B are 'Positive' or 'Borderline (equivocal)' as described in the Results Interpretation and Assay Criteria (see below), in which case the result is valid.

In the case of Invalid results, these should be reported as "Invalid" and it is recommended to collect a further sample and re-test the individual.

RESULTS INTERPRETATION AND ASSAY CRITERIA

Refer to the Quality Control section before applying the following criteria.

<u>NOTE</u>: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting T-SPOT.*TB* Refer to the most recent CDC guidance (http://www.cdc.gov/nchstp/tb) for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

Results for T-SPOT.*TB* are interpreted by subtracting the spot count in the Nil control well from the spot count in each of the Panels, according to the following algorithm:

- The test result is Positive if (Panel A-Nil) and/or (Panel B-Nil) ≥8 spots.
- The test result is Negative if both (Panel A-Nil) and (Panel B-Nil) ≤4 spots. This includes values less than zero.
- Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5, 6 or 7 spots should be considered Borderline (equivocal) and retesting by collecting another patient specimen is recommended.
- If the result is still Borderline (equivocal) on retesting with another specimen, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

The interpretation algorithm is described in the following Flow Diagram (Figure 3) and Tables 1-3. This algorithm also includes quality control criteria.

Figure 3 – Algorithm Flow Diagram

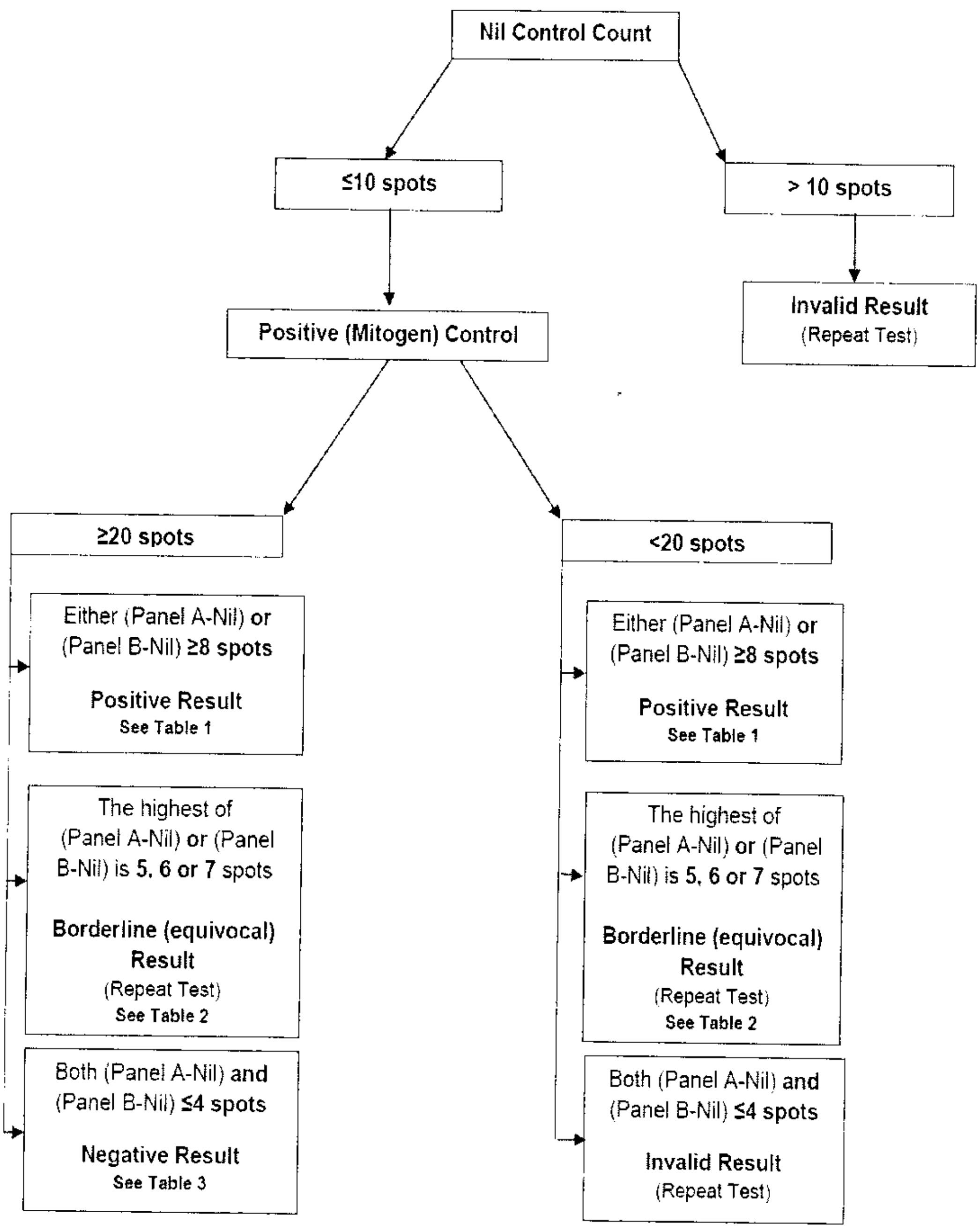


Table 1: Positive Interpretation: Either (Panel A-Nil) or (Panel B-Nil) ≥8 spots

Nil Control Well Count	Either Panel A **or** Panel B has the following number of spots†	Result Interpretation
0	≥8	**Positive**
1	≥9	**Positive**
2	≥10	**Positive**
3	≥11	**Positive**
4	≥12	**Positive**
5	≥13	**Positive**
6	≥14	**Positive**
7	≥15	**Positive**
8	≥16	**Positive**
9	≥17	**Positive**
10	≥18	**Positive**
>10 spots	n/a	**Invalid******

† *Note: The highest Panel-Nil spot count is to be used to determine the test outcome.*

Table 2: Borderline (equivocal) Interpretation: The highest of (Panel A-Nil) or (Panel B-Nil) is 5, 6 or 7 spots

Nil Control Well Count	The highest of Panel A **or** Panel B has the following number of spots	Result Interpretation
0	5, 6, or 7	**Borderline (equivocal)***
1	6, 7, or 8	**Borderline (equivocal)***
2	7, 8, or 9	**Borderline (equivocal)***
3	8, 9, or 10	**Borderline (equivocal)***
4	9, 10, or 11	**Borderline (equivocal)***
5	10, 11, or 12	**Borderline (equivocal)***
6	11, 12, or 13	**Borderline (equivocal)***
7	12, 13, or 14	**Borderline (equivocal)***
8	13, 14, or 15	**Borderline (equivocal)***
9	14, 15, or 16	**Borderline (equivocal)***
10	15, 16, or 17	**Borderline (equivocal)***
>10 spots	n/a	**Invalid****

Table 3: Negative Interpretation: Both (Panel A-Nil) and (Panel B-Nil) ≤4 spots

Nil Control Well Count	Both Panel A and Panel B has the following number of spots	Result Interpretation
0	≤4	**Negative**
1	≤5	**Negative**
2	≤6	**Negative**
3	≤7	**Negative**
4	≤8	**Negative**
5	≤9	**Negative**
6	≤10	**Negative**
7	≤11	**Negative**
8	≤12	**Negative**
9	≤13	**Negative**
10	≤14	**Negative**
>10 spots	n/a	**Invalid****

* *Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5,6 or 7 spots should be considered Borderline (equivocal) and retesting by collecting another patient specimen is recommended.*

*** In the case of Invalid results, these should be reported as "Invalid" and it is recommended to collect another sample and re-test the individual.*

7. LIMITATIONS

- Deviations from the instructions for use in this package insert may yield erroneous results.
- Incorrect performance of the assay may cause false positive or false negative responses.
- A false negative result can be caused by incorrect blood sample collection or improper handling of the specimen, affecting lymphocyte function.
- The performance of T-SPOT.*TB* has not been adequately evaluated with specimens from individuals younger than age 17 years, in pregnant women, and in patients with hemophilia.
- A false positive result was obtained for T-SPOT.*TB* when tested in subjects with *M. xenopi, M. kansasii and M. gordonae*. While ESAT-6 and CFP10 antigens are absent from BCG strains of *M. bovis* and from most environmental mycobacteria, it is possible that a positive T-SPOT.*TB* result may be due to infection with *M. kansasii, M. szulgai, M. gordonae* or *M. marinum*[4]. Alternative tests would be required if these infections are suspected.
- Results from T-SPOT.*TB* testing must be used in conjunction with each individual's epidemiological history, current medical status and results of other diagnostic evaluations.
- A negative test result does not exclude the possibility of exposure to, or infection with, *M. tuberculosis.* Patients with recent exposure to TB infected individuals exhibiting a negative T-SPOT.*TB* result should be considered for retesting within 6 weeks or if other relevant clinical symptoms indicate possible infection.
- A positive test result does not rule in active TB disease; other tests should be performed to confirm the diagnosis of active TB disease such as sputum smear and culture, PCR and chest radiography.
- T-SPOT.*TB* test has not been evaluated in subjects who have received > 1 month of anti-TB therapy.
- Refrigerated and frozen samples are not recommended for use with T-SPOT.*TB* test.

8. EXPECTED VALUES

The range of spot counts observed in response to the control antigens and the TB antigens that have been observed in clinical trials is shown in Figures 4a-c.

Figure 4a: Histogram of nil control responses from all study subjects

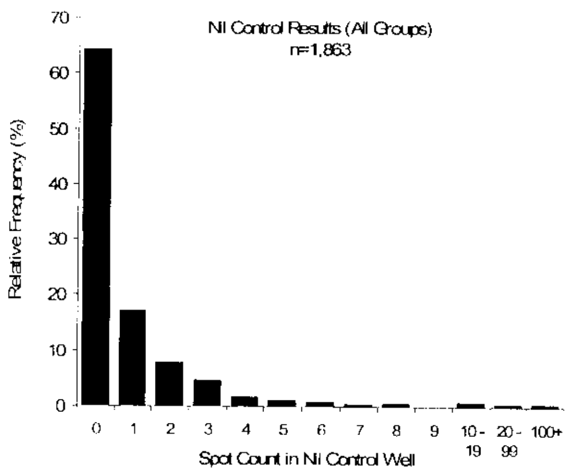


Figure 4b: Histogram of positive control responses from all study subjects

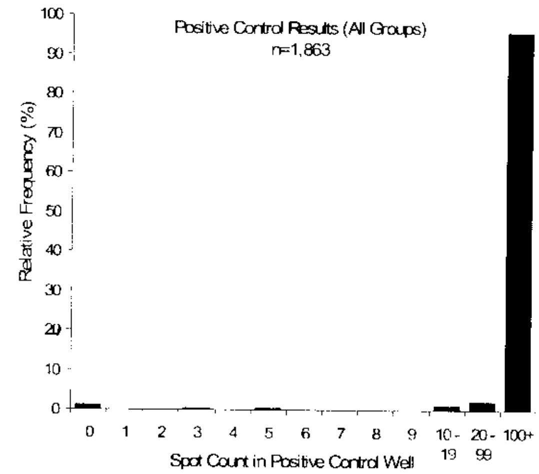
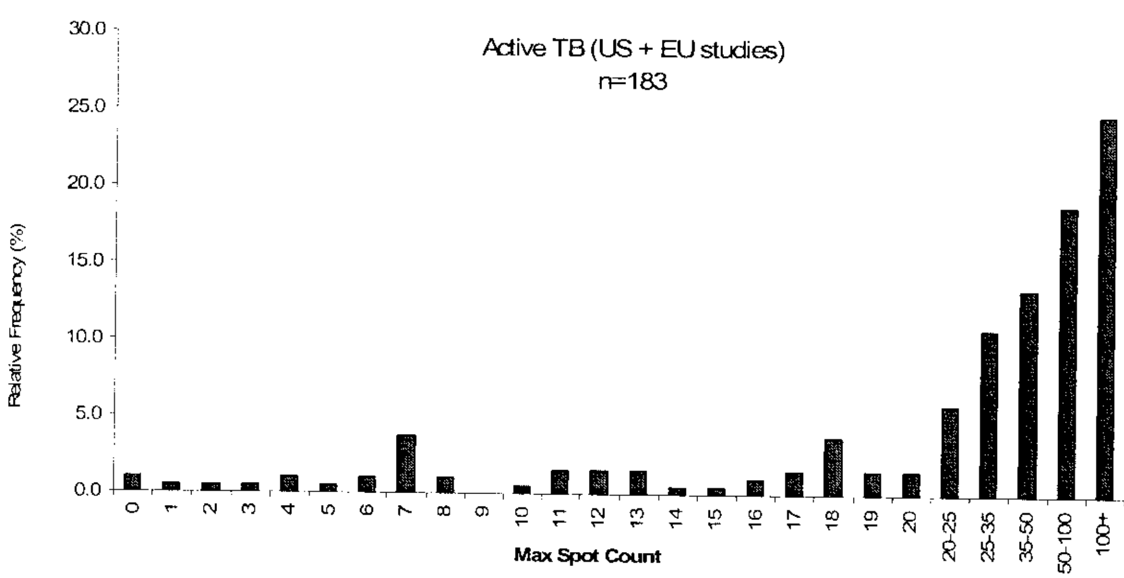


Figure 4c: Graph showing distribution of spot count in those with confirmed active TB. 'Max spot count' is the maximum (panel minus nil) response of either Panel A or Panel B



9. PERFORMANCE CHARACTERISTICS

ASSAY PERFORMANCE CHARACTERISTICS

The minimal detectable unit of response of the T-SPOT.*TB* assay is one spot.

Interference from heterophilic antibodies or intrinsic IFN-γ in the blood sample is minimized by the separation and washing of the PBMC fraction from whole blood. This potentially removes background amounts of IFN-γ, other interfering plasma moieties, hemoglobin and any heterophilic antibodies.

Cytokines expected to be produced by leucocytes, including IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNFα, IFN-α, and IFN-β were examined for cross-reactivity with the antibody pair used in the T-SPOT.*TB* assay. Results demonstrated that the antibody pair used in the T-SPOT.*TB* assay did not show evidence for cross-reactivity with other cytokines.

Intra-assay variability was analyzed by comparing the T-SPOT.*TB* assay run on the same plate by the same operator. Experiments were carried out by three operators on nine plates which resulted in a range of % CVs representative of the inherent variation in the test. The range that was obtained for the high spot counts (210.40 ± 11.59) was between 2.21% - 7.7% CV (mean % CV = 4.43), mid-range spot counts (71.17 ± 8.47) gave a range of 6.57% - 16.49% CV (mean % CV = 11.0%), whereas spot counts close to the cut-off (mean spot count = 5.71 ± 1.25) gave a mean % CV = 21.97%.

Inter-assay precision data were collected, where three kit lots were used by three different operators to run the same three samples on six occasions. The coefficient of variation measured across the three samples, three operators and three lots was 3.68% for samples giving a mean spot count of 210.40. For spot counts close to the T-SPOT.*TB* cut-off, the inter-assay variation was 24.95%. For moderate spot levels, the mean %CV was 13.86%. The results for the %CV were consistent for each of the batches tested.

Inter-operator reproducibility was assessed using three operators and one plate each from three kit batches. The variation observed between operators was 3.64%-5.76% CV.

Two separate inter-laboratory experiments were run wherein each case two tubes of blood were taken from the same patient and processed in parallel at the two sites. Results are shown in Table 4. For subjects with either positive or negative results at all sites, overall agreement was 95%.

Table 4 Results from inter-laboratory experiments

	Site 2		
Site 1	**Positive**	**Borderline (equivocal)**	**Negative**
Positive	13 (31.7%)	0	0
Borderline (equivocal)	0	0	1 (2.4%)
Negative	0	1 (2.4%)	26 (63.4%)

Samples (n=41) were run in parallel at Site 1 (OI) and one other site

The assay cutoff value was determined using Receiver Operating Characteristic (ROC) curve analysis[12]. A cut-off value of ≥6 spots maximized assay sensitivity and specificity.

SUMMARY OF CLINICAL DATA

A pivotal clinical study was designed to establish the clinical performance of the T-SPOT.*TB* test in both culture confirmed active TB disease and in potentially latent TB infection in populations stratified by risk of exposure to *M. tuberculosis*. A total of 2355 subjects were enrolled in the pivotal study; using 11 study sites as detailed in Table 5. Of the 2355 enrolled subjects, 492 samples did not meet the study criteria, leaving 1863 subjects available for analysis.

The 492 samples were excluded for the following reasons: 14 no informed consent/possible coercion, 11 excluded by site, 13 duplicate enrollments, 59 no blood sample collected, 115 insufficient sample collected to run T-SPOT.*TB* assay, 66 laboratory deviations in performing the T-SPOT.*TB* test, 18 no risk group assigned (incomplete data) and 196 without a TST result (121 recorded as no TST administered or no result provided, 67 with no record of the TST or missing medical records, 6 with a TST result from greater than a year prior to the study and no current result, 1 non-return for the reading of the TST result, 1 TST given post enrollment).

The study population as a whole was intended to include subjects from all major risk groups indicated for screening for TB infection according to guidelines from the CDC[2]. The performance of T-SPOT.*TB* was assessed in populations where the TST may likely give false-positive results (e.g. patients exposed to non-tuberculous mycobacteria and those who have previously received the BCG vaccination)[13]. In addition, the performance of T-SPOT.*TB* was assessed in populations where the TST may likely give false-negative results (e.g., patients of very young or old age and patients with various types of immunosuppression) and who may be at elevated risk of progression of latent TB infection to active TB disease[2,14-17]. All TST results were scored using 5, 10 or 15mm cutoffs according to CDC/ATS guidance[1].

Subjects from the pivotal study were classified into five main groups for analysis. The allocation of all patients from the pivotal study is detailed in Figure 5.

- Group 1 – Active TB (n=105)

The sensitivity of the T-SPOT.*TB* test was estimated from subjects where it was known that active, culture confirmed TB infection was present. Only subjects with positive culture confirmed results were included in this group. It was not a requirement for this group to have a TST result. 69 subjects were from Brazil, and 36 remaining subjects were from three Texas sites. In addition, results from studies in Germany (n=34), Italy (n=22) and the UK (n=28) were included into this Group for sensitivity calculations only. Thus, the sensitivity calculations were based on a combined group of 189 subjects, of which 6 T-SPOT.*TB* results were invalid, leaving 183 samples for analysis. Results are presented in Table 7.

- Group 2 – Low Risk Controls (n=311)

The specificity of the T-SPOT.*TB* test was estimated from subjects that were presumed as much as possible to have a lower probability of TB infection. "Low risk" subjects were selected on the basis of the absence of clinical, epidemiological and diagnostic risk factors for TB infection. A negative TST result was a requirement for this group.

Although common risk factors for TB infection were assessed in these subjects, a complete epidemiological background on each participant could not be obtained and, as such, this group may have contained subjects who had a genuine TB or NTM infection. This group was used to estimate specificity of T-SPOT.*TB*.

Factors used to determine a low risk of TB infection, based on patient self report, was the absence of any of the following factors:

- More than 3 months spent living in a TB endemic country (TB prevalence > 40/100,000)
- Occupational history of work in a high risk setting, e.g., TB laboratory, health care worker
- Time spent in a high risk environment, e.g., jail, nursing home or homeless shelter
- Known contact with TB Index case
- IV Drug use
- Heavy alcohol use
- Known Non-tuberculous (NTM) infection
- HIV infection or other immunosuppressive conditions

- History of having had TB or taken TB medication
- Radiological or microbiological test results consistent with TB infection
- Positive TST result

Of the 311 results, 5 had invalid T-SPOT.*TB* results leaving 306 results for analysis. Results are presented in Table 8.

- Group 3 – LTBI Suspects (n=1403)

This group, the largest in the study, included candidates for routine screening for LTBI infection according to the prevailing CDC guidelines for screening of risk groups. This group contained a broad mix of subjects screened for TB infection at varying degrees of risk of exposure (for example: recent contacts of known source cases, prison inmates) and risk of progression (for example: those with HIV infection, young children, the elderly, those with immunosuppressive conditions). This group was used to demonstrate the number of positive T-SPOT.*TB* results relative to TST results in these populations.

Of the 1403 results, 55 had invalid T-SPOT.*TB* results leaving 1348 results for analysis. Results are presented in Table 10.

- Group 4 – NTM (n=19)

This group contained a small group of subjects with known Non-Tuberculosis Mycobacterial (NTM) infections. [Note that these are also sometimes called Mycobacteria Other Than Tuberculosis (MOTT) or Atypical mycobacteria]. The group includes subjects with recently diagnosed (within previous 12 months) NTM infection, or those who were diagnosed more than 12 months previously but listed by the enrolling physician as having an active ongoing infection. TST was not a requirement, but was sometimes included in their normal clinical care. This group was used to demonstrate the estimated the cross reactivity of T-SPOT.*TB* in NTM infection. Of the 19 results, 1 was invalid by T-SPOT.*TB*, leaving 18 results available for analysis. Results are presented in Table 11.

- Group 5 – Unconfirmed Active TB (n=25)

Among those subjects recruited with active TB; 25 subjects were diagnosed clinically without culture-confirmation. TST was not consistently performed. Of the 25 results, 1 was invalid by T-SPOT.*TB* leaving 24 results for analysis. Results are presented on page 26.

A summary table of the number of subjects (after exclusions) by site comprising each group in the clinical study is shown in Table 5:

Table 5 – Summary of subjects comprising each clinical study group by site

Study site locations and primary enrolment populations in pivotal study	Group 1, Sensitivity calculations	Group 2, Specificity calculations	Group 3, LTBI Suspects	Group 4, NTM infection	Group 5, Unconfirmed Active TB	*Total number tested
Prison inmates, *TX*	7	0	462	5	3	477
Active TB patients *Brazil*	69	0	0	0	0	69
TB contacts *New York*	0	3	183	2	0	188
Children attending TB clinic, *TX*	12	13	184	1	18	228
HIV patients with suspected TB, *TX*	17	0	17	1	3	38
End Stage Renal Disease patients, *Canada*	0	0	195	0	1	196
Patients attending HIV clinic, *GA*	0	0	227	0	0	227
Naval Recruits screened on recruitment to Military, *IL*	0	294	52	0	0	346
Patients infected with NTMs, *NH*	0	1	2	10	0	13
Rheumatoid Arthritis patients on anti-TNFα therapy, *Canada*	0	0	34	0	0	34
Rheumatoid Arthritis patients on immunosuppressive therapies, *MA*	0	0	47	0	0	47
Total	105	311	1403	19	25	1863

**Includes invalid results*

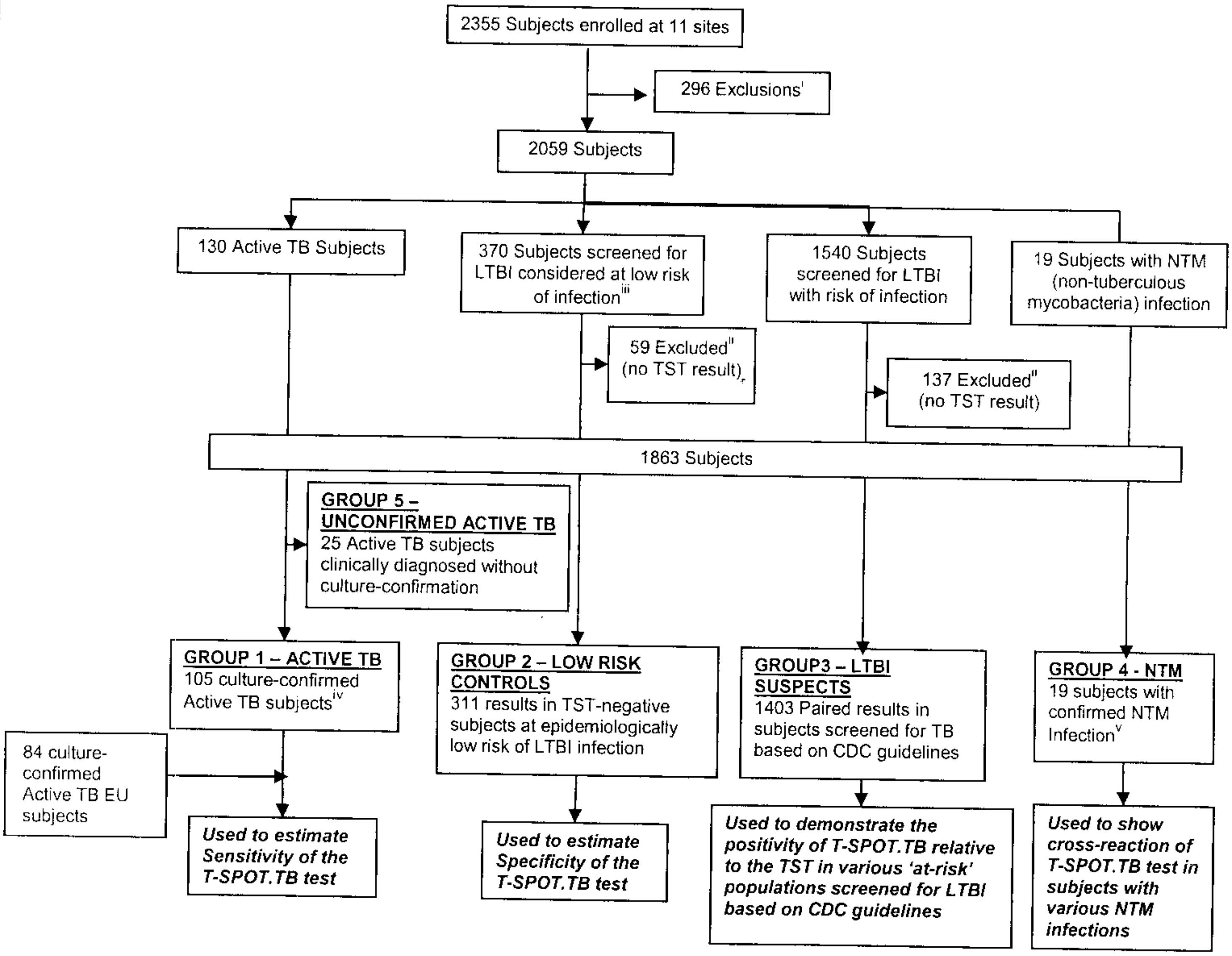
Demographic and key epidemiologic information for all subjects in the pivotal study by group are shown in Table 6.

Table 6 - Summary of pivotal study subject demographics by group

Gender	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**All Groups**
Males	62	260	917	11	13	1263
Females	43	50	483	8	12	596
Gender unknown/not recorded	0	1	3	0	0	4
Place of birth						
US born	20	299	687	18	15	1039
Foreign born	16	10	488	1	10	525
No data	69	2	228	0	0	299
BCG vaccination history						
BCG vaccinated	11	1	290	1	8	311
Not BCG vaccinated	22	212	783	17	15	1049
Unknown/not recorded	72	98	330	1	2	503
Ethnic group						
White	4	213	391	10	3	621
Black	8	40	532	6	1	587
Hispanic	91	34	335	2	16	478
Middle East / Indian	0	2	35	0	1	38
Asian	2	7	87	0	2	98
Native American / Alaskan	0	5	3	1	0	9
Hawaiian / Pacific Islander	0	3	6	0	0	9
Other	0	5	11	0	2	18
Ethnicity unknown/not recorded	0	2	3	0	0	5
Age group						
0 to 2 years	2	0	37	1	1	41
>2 to 5 years	1	2	41	0	6	50
6 to 17 years	6	9	78	0	11	104
Age not recorded	0	0	1	0	0	1
Age Range	0.4–69 years	3–65 years	0.08-93 years	1.83-77 years	2–52 years	0.08 – 93 years
0 to 17 years (children)	9	11	156	1	18	195
18 to 64 years (adult)	94	299	1117	14	7	1531
65+ years (elderly)	2	1	129	4	0	136
TOTAL	**105**	**311**	**1403**	**19**	**25**	**1863**

The Flow Diagram shown in Figure 5 below depicts subject accountability for all subjects enrolled in the pivotal study. The diagram also defines the intended analysis for each group evaluated in the study.

Figure 5 – Flow chart showing classification & usage of all 2355 subjects enrolled into the pivotal clinical trial.



i. 296 excluded samples were for the following reasons: 14 no informed consent/possible coercion, 11 excluded by site, 13 duplicate enrollments, 59 no blood sample collected, 115 insufficient sample collected to run T-SPOT.TB assay, 66 laboratory deviations in performing the T-SPOT.TB test, 18 no risk group assigned (incomplete data).

ii. 196 without a TST result (121 recorded as no TST administered or no result provided, 67 with no record of the TST or missing medical records, 6 with a TST result from greater than a year prior to the study and no current result, 1 non-return for the reading of the TST result, 1 TST given post enrollment).

iii. "Low risk" subjects were selected on the basis of the absence of clinical, epidemiological and diagnostic risk factors for TB infection.

iv. Subjects who had been receiving anti-TB therapy for greater than one month were excluded from this group in case of any interactions between the cure of the patient and test results. It was not a requirement in these groups to have a TST result for every patient.

v. Non-tuberculous mycobacterial (NTM) infection includes subjects with recently diagnosed (within previous 12 months) NTM infection, or those who were diagnosed more than 12 months previously but listed by the enrolling physician as having an active ongoing infection. TST was not a requirement.

Sensitivity Results (Pivotal Study Group 1 and European Study Subjects)

Table 7 – Sensitivity Results in pivotal study Group 1 patients and European study subjects

Source of culture-confirmed samples		T-SPOT.*TB*					Total	Sensitivity [95% CI]
		Positive	Borderline (equivocal)			Negative		
		≥8 spots	7 spots	6 spots	5 spots	≤4 spots		
US		31	3	0	0	2	36	94.4% (34/36) [81.3-99.3%]
BRAZIL		60	1	1	1	4	67	92.5% (62/67) [83.4-97.5%]
EU	GERMANY	31	1	0	0	1	33	97.0% (32/33) [84.2-99.9%]
	ITALY	19	1	0	0	0	20	100.0% (20/20) [86.1-100.0%]
	UK	25	1	1	0	0	27	100.0% (27/27) [89.5-100.0%]
TOTAL		**166**	**7**	**2**	**1**	**7**	**183***	**95.6% (175/183) [91.6-98.1%]**

* Out of the 189 total samples, 6 results were invalid: 1 subject with high background, 4 subjects with high Nil Control responses, 1 subject with low positive (mitogen) control response. Invalid results were excluded from calculations, leaving 183 samples for analysis.

Using the prespecified cutoff of ≥6 spots, the estimated sensitivity of T-SPOT.*TB* was 95.6% (175/183) [95%CI 91.6%-98.1%]

If all the borderline (equivocal) results are considered all positive or all negative, the estimated sensitivity of T-SPOT.*TB* was either 96.2% (176/183) or 90.7% (166/183), respectively.

Specificity Results (Group 2)

Table 8 –Specificity Results

US low risk samples	T-SPOT.*TB*					Total	Specificity [95% CI]
	Positive	Borderline (equivocal)			Negative		
	≥8 spots	7 spots	6 spots	5 spots	≤4 spots		
TOTAL	**3**	**1**	**5**	**7**	**290**	**306***	**97.1% (297/306) [94.5-98.7%]**

*Out of the 311 total samples, 5 results were invalid: 1 subject with high background, 1 subject with high Nil Control responses, 3 subjects with low positive (mitogen) control responses. Invalid results were excluded from calculations, leaving 306 samples for analysis.

Using the prespecified cutoff of ≥6 spots, the estimated specificity of T-SPOT.*TB* was 97.1% (297/306) [95%CI 94.5%-98.7%]

If all the borderline (equivocal) results are considered all positive or all negative, the estimated specificity of T-SPOT.*TB* was either 94.8% (290/306) or 99.0% (303/306), respectively.

Results in Subjects in ATS/CDC Risk Groups (Group 3)

There were 1403 subjects allocated to Group 3 who had a total of 1889 ATS/CDC risk factors. A number of BCG vaccinated and foreign-born individuals were included, which is consistent with the epidemiology of TB in the US[18]. A wide age range of subjects were also included.

Table 9 shows a breakdown of the numbers of Group 3 subjects, recruited within each ATS/CDC Risk Group and other key epidemiologic information. The table shows the numbers of patients within Group 3 who had each risk factor. Patients may have had more than one risk factor concurrently and therefore counted more than once in different rows of this table.

Table 9. Summary of subjects in Group 3 falling into ATS/CDC risk groups. Note that some subjects may have had multiple coincident conditions and therefore counted more than once in this table.

ATS/CDC Risk Group	Subjects with one or more of each risk factor	% of 1403 Group 3 subjects with one or more of each risk factor
HIV-positive persons	328	23.4%
Recent contacts of TB patients	229	16.3%
Patients with fibrotic changes on chest radiograph consistent with prior TB	26	1.9%
Patients with organ transplants and other immunosuppressed patients (receiving the equivalent of ≥15 mg/d of prednisone for 1 mo or more)[i]	122	8.7%
Recent immigrants (i.e., within the last 5 yr) from high prevalence countries	41	2.9%
Injection drug users	97	6.9%
Residents and employees of the following high-risk congregate settings: prisons and jails, nursing homes and other long-term facilities for the elderly, hospitals and other health care facilities, residential facilities for patients with acquired immunodeficiency syndrome (AIDS), and homeless shelters	613	43.7%
Mycobacteriology laboratory personnel	5	0.4%
Silicosis	0	0.0%
Diabetes mellitus	108	7.7%
Chronic renal failure (End-stage renal disease)	195	13.9%
Hematologic disorders	5	0.4%
Other specific malignancies [ii]	23	1.6%
Gastrectomy, and jejunoileal bypass	4	0.3%
Children younger than 4 yr of age or infants, children, and adolescents exposed to adults at high-risk	93	6.6%

i. Subjects taking the following drugs were included in this cohort: anti-TNF-alpha, steroids, transplant recipients, adalimumab, azathioprine, ciclosporin, etanercept, infliximab, leflunomide, methotrexate, mycophenolate mofetil, prednisone, sulfasalazine, tacrolimus

ii. Defined as any participant undergoing cancer chemotherapy

All groups indicated for screening according to prevailing CDC guidelines were represented in Group 3 as shown in Table 10 with the exception of silicosis patients, There is published data that shows the comparative results of T-SPOT.*TB* and TST for a group of silicosis subjects[19].

Results comparing the T-SPOT.*TB* test with the TST are shown in Table 10 below. The TST cut-off utilized for Group 3 was selected based on CDC recommendations for classification of tuberculin reaction.

Table 10. Summary of results for Group 3. All 55 Invalid assays excluded.

Group 3 (LTBI Suspects)		T-SPOT.*TB*					Total
		Positive	Borderline (equivocal)			Negative	
		≥8 spots	7 spots	6 spots	5 spots	≤4 spots	
TST	Positive	166	6	4	9	118	303
	Negative	116	23	13	25	868	1045
	TOTAL	282	29	17	34	986	1348

55 subjects were invalid, 9 had high background, 22 had high Nil Control responses, and 24 had low positive (mitogen) control responses. The invalid results were excluded from calculations.

Using the prespecified cutoff of ≥6 spots, the T-SPOT.*TB* results were positive in 24.3% (328/1348) and the TST was positive in 22.5% (303/1348) of Group 3 subjects. If all borderline (equivocal) results are considered either positive or negative, the percentage of T-SPOT.*TB* positive results was either 26.9% (362/1348) or 20.9% (282/1348), respectively.

The data from Group 3 were assessed to determine agreement of results between T-SPOT.*TB* and TST. Based on the prespecified cut-off of ≥6 spots, results are as follows:

- Overall Agreement = 79.3% (1069/1348) [95%CI 77.0-81.4%]
- Positive Agreement = 58.1% (176/303) [95%CI 52.3-63.7%]
- Negative Agreement = 85.5% (893/1045) [95%CI 83.2-87.5%]

If all the borderline (equivocal) results were considered positive:

- Overall Agreement = 78.1% (1053/1348) [95%CI 75.8-80.3%]
- Positive Agreement = 61.1% (185/303) [95%CI 55.3-66.6%]
- Negative Agreement = 83.1% (868/1045) [95%CI 80.7-85.3%]

If all the borderline (equivocal) results were considered negative:

- Overall Agreement = 81.2% (1095/1348) [95%CI 79.0-83.3%]
- Positive Agreement = 54.8% (166/303) [95%CI 49.0-60.5%]
- Negative Agreement = 88.9% (929/1045) [95%CI 86.8-90.7%]

An exploratory multiple logistic regression was performed to investigate association of test results and selected risk factors (gender, age, ethnicity, BCG vaccinated, immunocompromised, born in a TB endemic country, contact to infectious source case and history of prior TB infection status) and was conducted separately for the T-SPOT.*TB* and TST. The analyses were based on the 963 subjects in Group 3 with complete data for all of the variables included in the model. Therefore, 385 patients with incomplete data were not included. A positive T-SPOT.*TB* test was based on the prespecified cutoff of ≥6 spots. Invalid T-SPOT.*TB* results were not included. The same dataset was used for both T-SPOT.*TB* and TST. After controlling for the other variables in the model, positive results for both T-SPOT.*TB* and TST were significantly associated with history of prior TB infection. A positive result for T-SPOT.*TB* was significantly associated with contact to an infectious source and birth in a TB endemic country. However, a positive result for TST was not associated with those variables. A positive TST was associated with BCG vaccination; while, no association was observed between T-SPOT.*TB* results and BCG vaccination. A negative TST was associated with being immunocompromised; while, no association was observed between T-SPOT.*TB* result and immunocompromised status. A more positive TST results were observed among children (5-17 yrs) than among adults (18 -64 yrs); while, T-SPOT.*TB* results were not impacted by age.

Results in NTM infected (Group 4)

The TST is known to cross-react among those with Non-Tuberculous Mycobacterial infections[1,14], the most common of which is *M. avium*. Due to the use of antigens ESAT-6 and CFP10 that are not present in *M. avium*; T-SPOT.*TB* is not expected to cross-react in patients infected with this NTM. 12 subjects were identified with *M. avium* infection; none were positive with T-SPOT.*TB*. T-SPOT.*TB* results for subjects infected with *M. avium* and other Non-Tuberculous Mycobacteria are shown in Table 11 below.

Table 11. T-SPOT.TB and TST results among 18 patients with confirmed Non-Tuberculous Mycobacterial (NTM) infection. 1 invalid T-SPOT.TB result was excluded from the results.

Species of NTM identified	Number	# T-SPOT.*TB* Positive	# TST Positive
M. avium	12	0	Not done
*M. xenopii**	1	1	1
M. kansasii	1	1	1
M. gordonae	4	4	4

There were no borderline (equivocal) results
*** Note that this study participant was known to have had contact to an infectious source case and was strongly suspected of also having LTBI.*

Results in Unconfirmed Active TB (Group 5)

Of the 25 patients in Group 5, one was invalid by T-SPOT.*TB* (low positive control), leaving 24 samples for analysis. Using the prespecified cutoff of ≥6 spots, 79.2% (19/24) were positive by T-SPOT.*TB*. If the one borderline (equivocal) result (7 spots) was considered negative, the positive rate by T-SPOT.*TB* would be 75.0% (18/24); if the one borderline (equivocal) result was considered positive, the positive rate by T-SPOT.*TB* would be 79.2% (19/24).

SUMMARY OF PERFORMANCE

Sensitivity

– An estimated sensitivity of 95.6% (175/183) [95%CI 91.6%-98.1%] in subjects with culture-confirmed TB disease, using the prespecified cut-off of ≥6 spots.

Specificity

– An estimated specificity of 97.1% (297/306) [95%CI 94.5%-98.7%] in subjects with low risk of TB infection, using the prespecified cut-off of ≥6 spots.

The T-SPOT.*TB* invalid rate was 3.4% (64/1863) in the overall study data.

10. TROUBLESHOOTING

This assay should be performed using the principles of Good Laboratory Practice and by strictly adhering to these Instructions for Use.

Borderline (equivocal) Results

Borderline (equivocal) results are those where the maximum of the two (Panel minus Nil) spot count results are within ±1 spots from the ROC-determined assay cutoff of ≥6 spots. Borderline (equivocal) results, although valid, are less reliable than results where the spot count is further from the cut-off. Retesting of the patient, using a new sample, is therefore recommended. If the result is still Borderline (equivocal) on retesting, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

Invalid Results

Invalid results are uncommon and may be related to the immune status of the individual being tested[12]. They may also be related to a number of technical factors, potentially resulting in "high background", "low mitogen", and "high nil" results such as:

- Use of inappropriate blood collection tubes
- Storage of blood greater than 8 hours prior to processing
- Storage of blood outside the recommended temperature range (18-25°C prior to processing)
- Contamination of the cell culture media

- Incomplete plate washing

Repeating the test using a new patient sample is recommended for invalid results. Technical documents are available covering key troubleshooting points. These are available by contacting Oxford Immnotec.

For Technical Support in the United States contact: 1 – 877 – 20-TSPOT (87768).

11. ABBREVIATIONS & GLOSSARY OF SYMBOLS

ATS	American Thoracic Society
BCG	Bacillus Calmette-Guerin
BCIP/NBT	5-bromo, 4-chloro, 3-indoylphosphate/nitroblue tetrazolium
CDC	Centers for Disease Control and Prevention
CFP10	Culture Filtrate Protein
CPT	Cell Preparation Tubes
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immnunospot Assay
ESAT-6	Early Secretory Antigenic Target
FDA	Food and Drug Administration
IFN-γ	Interferon gamma
LTBI	Latent TB Infection
NTM	Non Tuberculous Mycobacteria
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PPD	Purified Protein Derivative (also known as Tuberculin)
RCF	Relative Centrifugal Force
RPM	Revolutions per minute
TST	Tuberculin Skin Test
Yrs	Years

12. REFERENCES

1. American Thoracic Society. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. *Am J Respir Crit Care Med.*, 2000; 161: 1376-1395
2. CDC. Targeted Tuberculin Testing and Treatment of Latent Tuberculosis. MMWR weekly, June 09, 2000; 49(RR06): 1 – 54.
3. PMA P010033 supplement number S011. Quantiferon-TB Gold In Tube package insert. Downloaded from www.cellestis.com. Date accessed 5th March 2008.
4. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune- based diagnosis of tuberculosis. *Lancet* 2000;356:1099–1104.
5. Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999;284:1520–3.
6. Lalvani A. Spotting latent infection: the path to better tuberculosis control. *Thorax*, 2003; 58(11): 916-918.
7. Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P and Ottenhoff T. Antigenic equivalence of Human T Cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and Culture Filtrate Protein 10 and to mixtures of synthetic proteins. *Infection and Immunity*, 2000; 68(6): 3314-3321.
8. Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, Conlon CP, Pasvol G and Hill AVS. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T Cells. *Am. J. Respir. Crit. Care Med.*, 2001; 163: 824-828.
9. NCCLS. Performance of single cell immune response assays; approved guideline. NCCLS document I/LA26-A.
10. Meier T, Eulenbruch H-P, Wrighton-Smith P, Enders G and Regnath T. Sensitivity of a new commercial enzyme-linked immunospot assay (T SPOT-TB) for diagnosis of tuberculosis in clinical practice. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2005; 24: 529-536.
11. Köller MD, Kiener HP, Aringer M, Graninger WB, Meuer S, Samstag Y, Smolen JS. Functional and molecular aspects of transient T cell unresponsiveness: role of selective interleukin-2 deficiency. *Clin Exp Immunol.* 2003 May;132(2):225-31.
12. NCCLS. Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristic (ROC) Plots. *NCCLS document GP10.*

13. Huebner RE, Schein MF and Bass JB Jr. The tuberculin skin test. *Clin. Infect. Dis.,* 1993; 17: 968-975.
14. Huebner RM, Schein M, Hall C and Barnes S. Delayed-type hypersensitivity anergy in human immunodeficiency virus-infected persons screened for infection with *Mycobacterium tuberculosis. Clin. Infect. Dis.* 1994; 19: 26-32
15. Shankar MS,Aravindan AN, Sohal PM, Kohil HS, Sud K, Gupta KL, Sakhuja V and Jha V. The prevalence of tuberculin sensitivity and anergy in chronic renal failure in an endemic area: tuberculin test and the risk of post-transplant tuberculosis. Nephrol Dial Transplant. 2005; Dec; 20(12): 2720 – 4
16. Fang HC, Chou KJ, Chen CL, Lee PT, Chiou YH, Hung SY and Chung HM. Tuberculin skin test and anergy in dialysis patients of a tuberculosis-endemic area. Nephron. 2002; Aug; 91(4): 682 – 7
17. CDC. Anergy skin testing and tuberculosis preventive therapy for HIV-infected persons: revised recommendations. MMWR. 1997; 46(RR-15): 1 – 10
18. Zuber PL, McKenna MT, Binkin NJ, Onorato IM and Castro KG. Long-term risk of tuberculosis among foreign-born persons in the United States. *JAMA,* 1997; 278: 304-307.
19. Leung CC, Yam WC, Yew WW, Ho PL, Tam CM, Law WS, Wong MY, Leung M, Tsui D. Comparison of T-Spot.TB and tuberculin skin test among silicotic patients. *Eur Respir J.* 2007 Oct 24

13. CONTACT INFORMATION

Oxford Immunotec, Inc
2 Mount Royal Avenue, Suite 100
Marlborough, MA 01752, USA
Toll Free: 877-208-7768 (877-20T-SPOT)

Oxford Immunotec Ltd
94C Milton Park, Abingdon
Oxfordshire, OX14 4RY, UK
Tel: +44 (0) 1235 442796

For product support downloads and further technical information, please visit our website:
www.oxfordimmunotec.com

T-SPOT and the Oxford Immunotec logo are trademarks of Oxford Immunotec Limited
AIM-V, RPMI and GIBCO are trademarks of Invitrogen
CPT and Vacutainer are trademarks of Becton Dickinson
Ficoll-Paque is a trademark of GE Health Technologies
Tween is a trademark of ICI Americas

T-SPOT.*TB* is protected by the following patents and patents pending :
US 09/308,725, EP 941478, JP 1998-524410, AU 728357, CA 2272881, EP 1152012, AU 765013, US 7115361, US 09/830,839, EP 99952697.3, JP 2000-579635, ZA 2001-3356, US 7135280, EP 02726998.4, JP 2002-554719, AU 2002-219338, CA 2483236, CZ 2003/001866, IN 0105/DELNP/2003, NZ 526807, US 6290969, US 6338852, US 6350456, US 6458366, US 6962710, EP 850305, EP 851927, EP 1203817, JP 2001-517069, AU 727602, AU 756833, AU 753995, BR 9610262, BR 9610268, CA 2230885, CA 2230927, CN 1200146, CN 1200147, CZ 9800628, HU 9900902, IL 123506, NO 9800883, PL 325373, TR 9800411, ZA 9607394, ZA 9607395, US 5955077, EP 706571, JP 2001-515359, AU 682879, CA 2165949, NZ 267984, US 1999/132505, EP 928851, JP 2000-615041, AU 773268, CA 2372583.

T-SPOT.*TB* incorporates patented technology under license from the Statens Serum Institut, Copenhagen, Denmark, Isis Innovation Limited, Oxford, UK and the Public Health Research Institute, New York, USA and may only be used for human *in vitro* diagnostics.

Revision number: 1 Date of Issue: XXXXX

Oxford Immunotec

Harnessing the power of T cell measurement

T-SPOT.

Clinicians' Guide

Description of the Test

The immune response to infection with *Mycobacterium tuberculosis* is mediated predominantly through T cell activation. As part of this response, T cells are sensitized to *M. tuberculosis* antigens and the activated effector T cells, both CD4+ and CD8+; produce the cytokine interferon gamma (IFN-γ) when stimulated by these antigens[1-2]. The T-SPOT.*TB* test uses the enzyme-linked immunospot (ELISPOT) methodology to enumerate *M. tuberculosis*-sensitized T cells by capturing interferon-gamma (IFN-γ) in the vicinity of T cells from which it was secreted.[3] The test enumerates effector T cells responding to stimulation with a combination of peptides simulating ESAT-6 and CFP10 antigens. ESAT-6 and CFP10 are absent from all BCG strains and from most non-tuberculous mycobacteria with the exception of *M. kansasii, M. szulgai* and *M. marinum*[4-6]. In contrast, individuals infected with *M. tuberculosis* complex organisms (*M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti*) usually have T cells in their blood which recognize these and other mycobacterial antigens. Blood samples are collected into either sodium citrate or sodium heparin Vacutainer® CPT tubes (Becton Dickinson), or lithium heparin blood collection tubes and then the PBMCs are separated in the tube using the manufacturer's instructions. Blood samples should be processed within 8 hours.

Intended use and Suitable Populations

T-SPOT·*TB* is an in vitro diagnostic test for the detection of effector T cells that respond to stimulation by *Mycobacterium tuberculosis* antigens ESAT-6 and CFP-10 by capturing interferon gamma (IFN-γ) in the vicinity of T cells in human whole blood collected in sodium citrate or sodium or lithium heparin. It is intended for use as an aid in the diagnosis of *M. tuberculosis* infection.

T-SPOT.*TB* is an indirect test for *M. tuberculosis* infection (including disease) and is intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations.

T-SPOT.*TB* can be used for people who are being tested for TB infection, with the following limitations. The performance of T-SPOT.*TB* has not been adequately evaluated with specimens from individuals younger than age 17 years, in pregnant women and with hemophilia. T-SPOT.*TB* test has not been evaluated in subjects who have received > 1 month of anti-TB therapy.

A false positive result was obtained for T-SPOT.*TB* when tested in subjects with *M. xenopi, M. kansasii and M. gordonae.* While ESAT-6 and CFP10 antigens are absent from BCG strains of *M. bovis* and from most environmental mycobacteria, it is possible that a positive T-SPOT.*TB* result may be due to infection with *M. kansasii, M. szulgai, M. gordonae* or *M. marinum*[4]. Alternative tests would be required if these infections are suspected.

The T-SPOT.*TB* test has been tested in some patient groups indicated for screening for TB infection according to current ATS/CDC Guidance[7]: such as, human immunodeficiency virus (HIV) positive persons, recent contacts of TB case patients, residents and employees of high-risk congregate settings, chronic renal failure, children younger than 4 yr of age or infants, children, and adolescents exposed to adults at high-risk and immunosuppressed patients.

Interpretation of Results

NOTE: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting T-SPOT. *TB*. Refer to the most recent CDC guidance (http://www.cdc.gov/nchstp/tb) for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

The results from the T-SPOT.*TB* assay are ready the following day. Results for T-SPOT.*TB* are interpreted by subtracting the spot count in the Nil control well from the spot count in each of the Panels, according to the following algorithm:

The test result is Positive if (Panel A-Nil) and/or (Panel B-Nil) ≥8 spots.

A positive test result does not indicate active TB disease. Other tests should be performed to confirm the diagnosis of active TB disease such as sputum smear and culture, PCR and chest radiography.

The test result is Negative if both (Panel A-Nil) and (Panel B-Nil) ≤4 spots. This includes values less than zero.

A negative test result does not exclude the possibility of exposure to, or infection with, *M. tuberculosis*. Patients with recent exposure to TB infected individuals exhibiting a negative T-SPOT.*TB* result should be considered for retesting within 6 weeks or if other relevant clinical symptoms indicate possible infection.

The test result is Borderline (equivocal) when the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5, 6, or 7 spots and retesting by collecting another patient specimen is recommended.

If the result is still borderline (equivocal) on retesting with another patient specimen, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

Invalid test results should be retested by collecting another patient specimen.

Results of Clinical Trials

A pivotal clinical study was designed to establish the clinical performance of the T-SPOT.*TB* test in both culture confirmed active TB disease and in potentially latent TB infection in populations stratified by risk of exposure to *M. tuberculosis*. A total of 2355 subjects were enrolled in the pivotal study; using 11 study sites. Of the 2355 enrolled subjects, 492 samples did not meet the study criteria, leaving 1863 subjects available for analysis.

The performance of T-SPOT.*TB* was assessed in populations where the TST may likely give false-positive results (e.g. patients exposed to non-tuberculous mycobacteria and those who have previously received the BCG vaccination)[8]. In addition, the performance of T-SPOT.*TB* was assessed in populations where the TST may likely give false-negative results (e.g., patients of very young or old age and patients with various types of immunosuppression) and who may be at elevated risk of progression of latent TB infection to active TB disease[5,7,9-12]. All TST results were scored using 5, 10 or 15mm cutoffs according to CDC/ATS guidance[13].

Subjects from the pivotal study were classified into five main groups for analysis:

Group 1 – Active TB (n=105)

The sensitivity of the T-SPOT.*TB* test was estimated from subjects where it was known that active, culture confirmed TB infection was present. This group included subjects from Brazil and the U.S. (n = 105) as well as from Europe (n = 84) for a total of 189 subjects. Six results were invalid, leaving 183 for analyses.

An estimated sensitivity of 95.6% (175/183) [95%CI 91.6%-98.1%] was obtained in subjects with culture-confirmed TB disease, using the prespecified cut-off of ≥6 spots.

There were 10 (of 183) borderline (equivocal) results. If all the borderline (equivocal) results are considered all positive or all negative, the estimated sensitivity of T-SPOT.*TB* was either 96.2% (176/183) or 90.7% (166/183), respectively.

Group 2 – Low Risk Controls (n=311)

The specificity of the T-SPOT®.*TB* test was estimated from subjects that were presumed as much as possible to have a lower probability of TB infection. A negative TST was a requirement for this group. Five results were invalid, leaving 306 results for analyses.

An estimated specificity of 97.1% (297/306) [95%CI 94.5%-98.7%] was obtained in subjects with low risk of TB infection, using the prespecified cut-off of ≥6 spots.

There were 13 (of 306) borderline (equivocal) results. If all the borderline (equivocal) results are considered all positive or all negative, the estimated specificity of T-SPOT.*TB* was either 94.8% (290/306) or 99.0% (303/306), respectively.

Group 3 – LTBI Suspects (n=1403)

Group 3, the largest in the study, included candidates for routine screening for LTBI infection according to the prevailing CDC guidelines for screening of risk groups. This group contained a broad mix of subjects screened for TB infection at varying degrees of risk of exposure (for example: recent contacts of known source cases, prison inmates) and risk of progression (for example: those with HIV infection, young children, the elderly, those with immunosuppressive conditions). This group was used to demonstrate the number of positive T-SPOT.*TB* results relative to TST results in these populations. Fifty five results were invalid, leaving 1348 for analyses.

Using the prespecified cutoff of ≥6 spots, the T-SPOT.*TB* results were positive in 24.3% (328/1348) and the TST was positive in 22.5% (303/1348)

The data from Group 3 were assessed to determine agreement of results between T-SPOT.*TB* and TST. Based on the pre-specified cut-off of ≥6 spots, results are as follows:

- Overall Agreement = 79.3% (1069/1348) [95%CI 77.0-81.4%]
- Positive Agreement = 58.1% (176/303) [95%CI 52.3-63.7%]
- Negative Agreement = 85.5% (893/1045) [95%CI 83.2-87.5%]

There were 80 (of 1348) borderline (equivocal) results. If all the borderline (equivocal) results were considered positive:

- Overall Agreement = 78.1% (1053/1348) [95%CI 75.8-80.3%]
- Positive Agreement = 61.1% (185/303) [95%CI 55.3-66.6%]
- Negative Agreement = 83.1% (868/1045) [95%CI 80.7-85.3%]

If all the borderline (equivocal) results were considered negative:

- Overall Agreement = 81.2% (1095/1348) [95%CI 79.0-83.3%]
- Positive Agreement = 54.8% (166/303) [95%CI 49.0-60.5%]
- Negative Agreement = 88.9% (929/1045) [95%CI 86.8-90.7%]

Group 4 – NTM (n=19)

Group 4 contained a small number of subjects with known Non-Tuberculosis Mycobacterial (NTM) infections. This group was used to determine cross-reactivity of the assay. One result was invalid, leaving 18 results for analyses. Due to the use of antigens ESAT-6 and CFP10 that are not present in *M. avium*; T-SPOT.*TB* is not expected to cross-react in patients infected with this NTM. 12 subjects were identified with *M. avium* infection; none were positive with T-SPOT.*TB*.

Group 5 – Unconfirmed Active TB (n=25)

Among those subjects recruited with active TB; 25 subjects were diagnosed clinically without culture-confirmation (unconfirmed). One result was invalid, leaving 24 for analyses. Using the prespecified cutoff of ≥6 spots, 79.2% (19/24) were positive by T-SPOT.*TB*. If the one borderline (equivocal) result (7 spots) was considered negative, the positive rate by T-SPOT.*TB* would be 75.0% (18/24); if the one borderline (equivocal) result was considered positive, the positive rate by T-SPOT.*TB* would be 79.2% (19/24).

The T-SPOT.*TB* invalid rate was 3.4% (64/1863) in the overall pivotal study data.

References

1. Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P and Ottenhoff T. Antigenic equivalence of Human T Cell responses to *Mycobacterium tuberculosis*-specific RD1-encoded protein antigens ESAT-6 and Culture Filtrate Protein 10 and to mixtures of synthetic proteins. *Infection and Immunity*, 2000; 68(6): 3314-3321.
2. Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, Conlon CP, Pasvol G and Hill AVS. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T Cells. *Am. J. Respir. Crit. Care Med.*, 2001; 163: 824-828.
3. NCCLS. Performance of single cell immune response assays; approved guideline. NCCLS document I/LA26-A.
4. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune- based diagnosis of tuberculosis. *Lancet* 2000; 356: 1099–1104.
5. Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999; 284: 1520–3.
6. Lalvani A. Spotting latent infection: the path to better tuberculosis control. *Thorax*, 2003; 58(11): 916-918.
7. CDC. Targeted Tuberculin Testing and Treatment of Latent Tuberculosis. MMWR weekly, June 09, 2000; 49(RR06): 1 – 54.
8. Huebner RE, Schein MF and Bass JB Jr. The tuberculin skin test. *Clin. Infect. Dis.*, 1993; 17: 968-975.
9. Huebner RM, Schein M, Hall C and Barnes S. Delayed-type hypersensitivity anergy in human immunodeficiency virus-infected persons screened for infection with *Mycobacterium tuberculosis. Clin. Infect. Dis.* 1994; 19: 26-32
10. Shankar MS,Aravindan AN, Sohal PM, Kohil HS, Sud K, Gupta KL, Sakhuja V and Jha V. The prevalence of tuberculin sensitivity and anergy in chronic renal failure in an endemic area: tuberculin test and the risk of post-transplant tuberculosis. Nephrol Dial Transplant. 2005; Dec; 20(12): 2720 – 4
11. Fang HC, Chou KJ, Chen CL, Lee PT, Chiou YH, Hung SY and Chung HM. Tuberculin skin test and anergy in dialysis patients of a tuberculosis-endemic area. Nephron. 2002; Aug; 91(4): 682 – 7
12. CDC. Anergy skin testing and tuberculosis preventive therapy for HIV-infected persons: revised recommendations. MMWR. 1997; 46(RR-15): 1 – 10
13. American Thoracic Society. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. *Am J Respir Crit Care Med.,* 2000; 161: 1376-1395
14. Köller MD, Kiener HP, Aringer M, Graninger WB, Meuer S, Samstag Y, Smolen JS. Functional and molecular aspects of transient T cell unresponsiveness: role of selective interleukin-2 deficiency. *Clin Exp Immunol.* 2003 May;132 (2):225-31.

Contact

Oxford Immunotec, Inc.
2 Mount Royal Avenue, Suite 100
Marlborough, MA 01752, USA
Toll Free: 877-208-7768 (877-20T-SPOT)

Oxford Immunotec Ltd
94C Milton Park, Abingdon
Oxfordshire, OX14 4RY, UK
Tel: +44 (0) 1235 442796

Our website: www.oxfordimmunotec.com

Revision number: 1 Date of Issue: XXXXX

Oxford Immunotec

Harnessing the power of T cell measurement

T-SPOT.

TRAINING GUIDE

For *In Vitro* Diagnostic Use Only

This Visual Procedure Training Guide covers use of both:
T-SPOT.*TB 8* (Multi-use 8-Well Strip Plate Format. Catalogue number: TB.300)
T-SPOT.*TB 96* (Single-use 96-well Plate Format. Catalogue number: TB.200)

To be used in conjunction with the T-SPOT.*TB* Package Insert [PI-TB-US-V1]

Oxford Immunotec

Harnessing the power of T cell measurement

TG-TB-US-V1

INTRODUCTION

This training guide is intended to give guidance and information to the operator in preparing and running the T-SPOT.*TB* test. This guide explains sample collection, preparation of Peripheral Blood Mononuclear Cells (PBMCs) prior to running the assay, performing T-SPOT.*TB*, interpreting test results and troubleshooting.

Using this document

Instructions from the T-SPOT.*TB* package insert are provided in the top section of each relevant page. Additional information such as notes, corresponding visual aids and examples are shown in blue text beneath these instructions.

Table of contents

66

Reagent & Storage

MATERIALS PROVIDED

T-SPOT.*TB 8* (Multi-use 12 x 8-well strip version) contains:

1. . 1 microtiter plate: 96 wells, supplied as 12x 8-well strips in a frame, coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ).
2. 2 vials (0.8mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.8mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
   4.2 vials (0.8mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50μL) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT^{plus} solution.
7. CD containing the package insert

T-SPOT.*TB 96* (Single-use solid 96-well plate version) contains:

1. 1 microtiter plate: 96 wells coated with a mouse monoclonal antibody to the cytokine interferon gamma (IFN-γ).
2. 2 vials (0.7mL each) Panel A: contains ESAT-6 antigens, bovine serum albumin and antimicrobial agents.
3. 2 vials (0.7mL each) Panel B: contains CFP10 antigens, bovine serum albumin and antimicrobial agents.
4. 2 vials (0.7mL each) Positive Control: contains phytohemagglutinin (PHA), for use as a cell functionality control, bovine serum albumin and antimicrobial agents.
5. 1 vial (50μL) 200x concentrated Conjugate Reagent: mouse monoclonal antibody to the cytokine IFN-γ conjugated to alkaline phosphatase.
6. 1 bottle (25mL) Substrate Solution: ready-to-use BCIP/NBT^{plus} solution.
7. CD containing the package insert

STORAGE & STABILITY

Store the unopened kit at 2-8°C. The components of the kit are stable up to the expiration date printed on the kit box, when stored and handled under the recommended conditions. The kit must not be used beyond the expiration date on the kit label.

For T-SPOT.*TB 8*, store opened kit components at 2-8°C. Opened components must be used within 8 weeks of opening, such period ending no later than the expiration date on the kit label. **Avoid prolonged exposure of the Substrate Solution to light.**

T-SPOT.*TB 96* is a single-use kit and, once opened, all materials should be used immediately and not reused. Do not mix components between different kits.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

1. 8-well strip plate frame (available from Oxford Immunotec).
2. BLII cabinet (recommended).
3. Blood collection tubes, such as Vacutainer® CPT™ or heparinized tubes (Note: CPT tubes are available from Oxford Immunotec).
4. Ficoll (if not using CPT tubes)
5. A centrifuge for preparation of PBMCs (capable of at least 1800 RCF (g) and able to maintain the samples at room temperature (18-25°C)) if using density centrifugation methods to separate the PBMCs.
6. Equipment and reagents to enable counting of PBMCs; either manually using Trypan Blue (or other appropriate stain) and a hemocytometer on a microscope or automatically using a suitable hematology analyzer.
7. A humidified incubator capable of 37 ± 1°C with a 5% CO_2 supply.
8. An automatic microtiter plate washer or an 8 channel or stepper pipette to manually wash plates.
9. Adjustable pipettes to cover a range of volumes from 1-1000µl (such as four Gilson pipettes capable of delivering volumes of 1-10µL, 2-20µL, 20-200µL and 100-1000µL) and sterile pipette tips.
10. Sterile PBS solution: such as GIBCO™ 1x D-PBS (Invitrogen; catalogue number 14040-133).
11. Distilled or deionized water.
12. A means of visualizing the wells, or capturing a digital image of the well, such as a stereomicroscope, magnifying glass or plate imager to allow counting of spots.
13. Sterile cell culture medium such as GIBCO AIM-V™ (Invitrogen; catalogue number 31035-025 research grade). (Note: AIM-V media is available from Oxford Immunotec). **The use of this serum free medium for the incubation step is strongly recommended**. RPMI 1640 (Invitrogen; catalogue number 11875-093) may be used in the initial sample preparation steps only. It is recommended that cell culture media are stored in appropriate aliquots and excess material is discarded after use. **Cell culture media should be pre-warmed to 37°C before use with T-SPOT.*TB*.** To avoid problems with contaminated media, it can be helpful to dispense bottles of AIM-V or RPMI 1640 into smaller aliquots.

SPECIMEN COLLECTION & HANDLING

Package Insert Instructions For Use

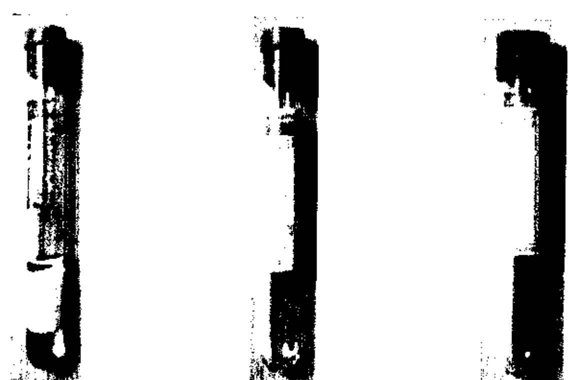
SPECIMEN COLLECTION & HANDLING

Individual laboratories should validate their procedures for collection and separation of PBMCs to obtain sufficient numbers. It is recommended that:

- Blood samples are collected into either sodium citrate or sodium heparin Vacutainer® CPT tubes (Becton Dickinson) and then the PBMCs separated in the tube using the manufacturer's instructions.
- Blood samples are collected into lithium heparin blood collection tubes with PBMCs being subsequently separated using standard separation techniques such as Ficoll-Paque®. Alternative methods to purify the PBMC fraction may be employed if desired. **Note EDTA tubes are not recommended.**
- Patient's cells can be pooled, if necessary to obtain sufficient cells from multiple tubes of blood which were collected and processed concurrently.

Training Notes and Visual Aids

Blood collection tubes which are suitable for use with T-SPOT.*TB* are highlighted in Figure 1.

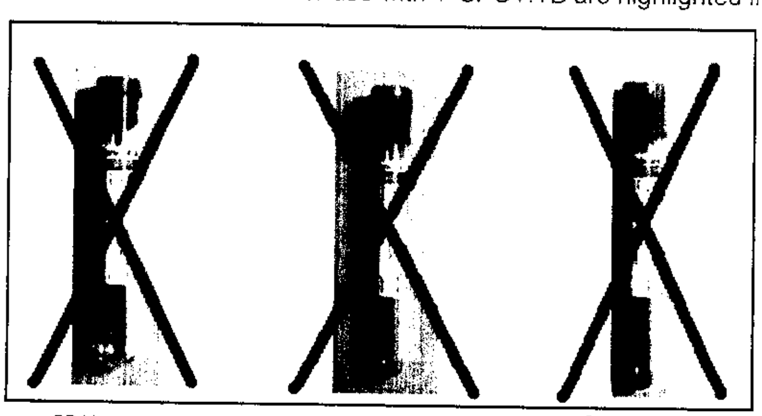


BD Sodium Citrate Vacutainer® CPT™ | BD Vacutainer® Lithium Heparin PST™ | Greiner Bio-One Lithium Heparin Vacuette®

Figure 1: Examples of blood collection tubes which are suitable for use with T-SPOT.*TB*.

Note: *Tubes from alternative suppliers that contain lithium heparin, sodium citrate or sodium heparin may also be used for specimen collection.*

Blood collection tubes which are not suitable for use with T-SPOT.*TB* are highlighted in Figure 2.



BD Vacutainer® Z | BD Vacutainer® CAT | BD Vacutainer® EDTA (K2E or K3E)

Figure 2: Examples of blood collection tubes which are unsuitable for use with T-SPOT.*TB*.

Note: *Tubes from alternative suppliers that do not contain lithium heparin, sodium citrate or sodium heparin should not be used for specimen collection*

Package Insert Instructions For Use

Typically for an immunocompetent patient, sufficient PBMCs to run the assay can be obtained from venous blood samples according to the following guidelines

- Adults and children 10 years old and over one 8mL or two 4mL tubes (CPT) or two lithium heparin 6mL tubes
- Children 2-9 years old one 4mL tube (both methods)
- Children up to 2 years old one 2mL pediatric tube (both methods)

Blood samples should be processed within 8 hours. Samples may be successfully stored for longer periods of time but users should validate this in their own setting[1,2]. **Whole blood samples should be maintained between 18°C and 25°C until processed** The instructions below give more information on specimen collection steps

1 Collect a blood sample according to the instructions supplied with the collection device The tube contents must be inverted (8 – 10 times) to ensure that the whole blood is mixed thoroughly with the anticoagulant Store collected blood at room temperature (18-25°C). **Do not refrigerate or freeze**

2 For CPT blood collection tubes, centrifuge 8mL CPT tubes at 1600 RCF(g) for 28 minutes or 4mL CPT tubes at 1800 RCF (g) for 30 minutes at room temperature (18-25°C) If using Ficoll-Paque™ Plus, dilute the blood with an equal volume of RPMI 1640 medium (1 part blood to 1 part RPMI) Layer carefully the diluted blood onto Ficoll-Paque Plus (2-3 parts diluted blood to 1 part Ficoll-Paque) and centrifuge at 1000 RCF (g) for 22 minutes at room temperature (18 25°C)

Training Notes and Visual Aids

Separating PBMCs by BD Vacutainer® Cell Preparation Tube (CPT™)

Collect blood in 4mL or 8mL CPTs following the manufacturer s instructions (Figure 3)

Centrifuge CPTs at 18-25°C

- 4mL - 1800 RCF (g) for 30 minutes
- 8mL - 1600 RCF (g) for 28 minutes

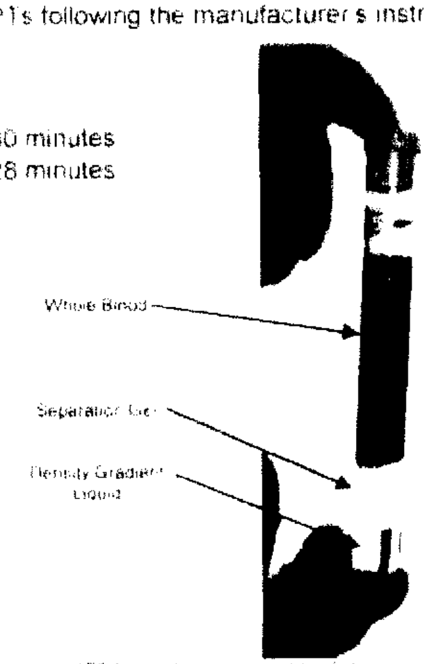


Figure 3 CPT filled with whole blood ready for centrifugation

Notes
RCF = Relative Centrifugal Force The term RCF and not RPM is used to separate aqueous solutions in the centrifuge
A refrigerated centrifuge is not essential if a refrigerated centrifuge is not available take additional care to ensure the rotor is well balanced as any slight vibration will cause a build up of heat which will alter the density of the gel layer in the CPT and the PBMCs will migrate into the red cell layer
To simplify patient identification after cell separation label tubes towards the top to allow easy viewing of labels above the Red Blood Cell and PBMC layers
Warning It is recommended that PBMC separation steps are performed in a BL II Safety Cabinet to protect the user and prevent contamination of the samples

<u>Isolation of PBMCs by Ficoll Extraction Method</u>

Collect appropriate volume of blood into lithium heparin tubes following the manufacturer's Instructions and dilute 1:1 with RPMI 1640 pre-warmed to 37°C. Carefully layer diluted blood sample onto Ficoll-Paque™ Plus (Figure 4) at a ratio of 3 volumes of diluted blood to 1 volume Ficoll-Paque Plus. Do not allow the layers to mix (Figure 5).

Centrifuge at 1000 RCF (g) for 22 minutes at room temperature (18-25°C).

Ensure the tubes are balanced before centrifugation in an aerosol-resistant bucket, with the brake off.

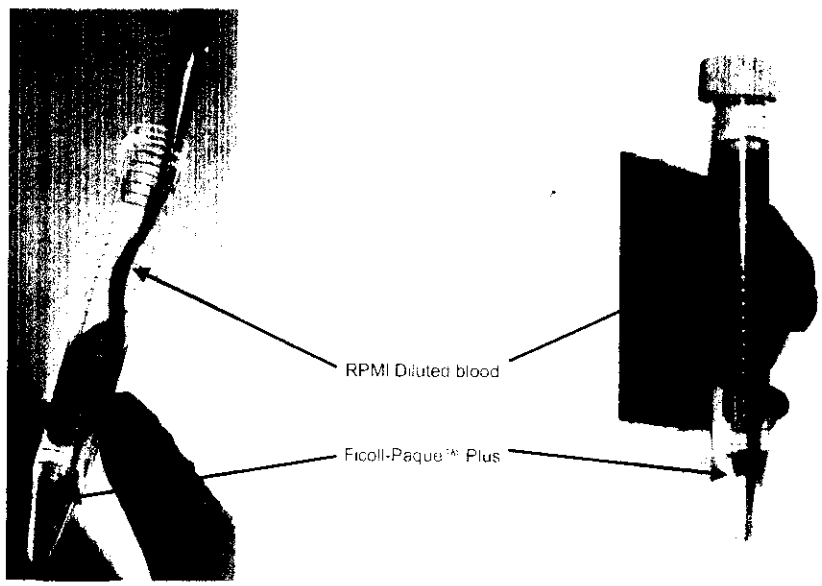


Figure 4 & 5. layering diluted whole blood on Ficoll-Paque and tube ready for centrifugation, respectively

Notes:

If a low cell yield is suspected then collect: 2x 8mL CPTs, 2x 6mL Lithium Heparin Vacutainer tubes or similar (see page 3). This will ensure sufficient PBMC yield to run the assay.

PBMC isolation can be achieved using CPTs or Ficoll gradients. Leucosep tubes (Greiner Bio-One) offer a time-saving approach to Ficoll gradients. These tubes contain a porous barrier that enables the blood sample to be poured onto the Ficoll gradient, thereby eliminating the need to gently layer the sample on Ficoll-Paque.

BD Cell Preparation Tube (CPT™) Ficoll Extraction

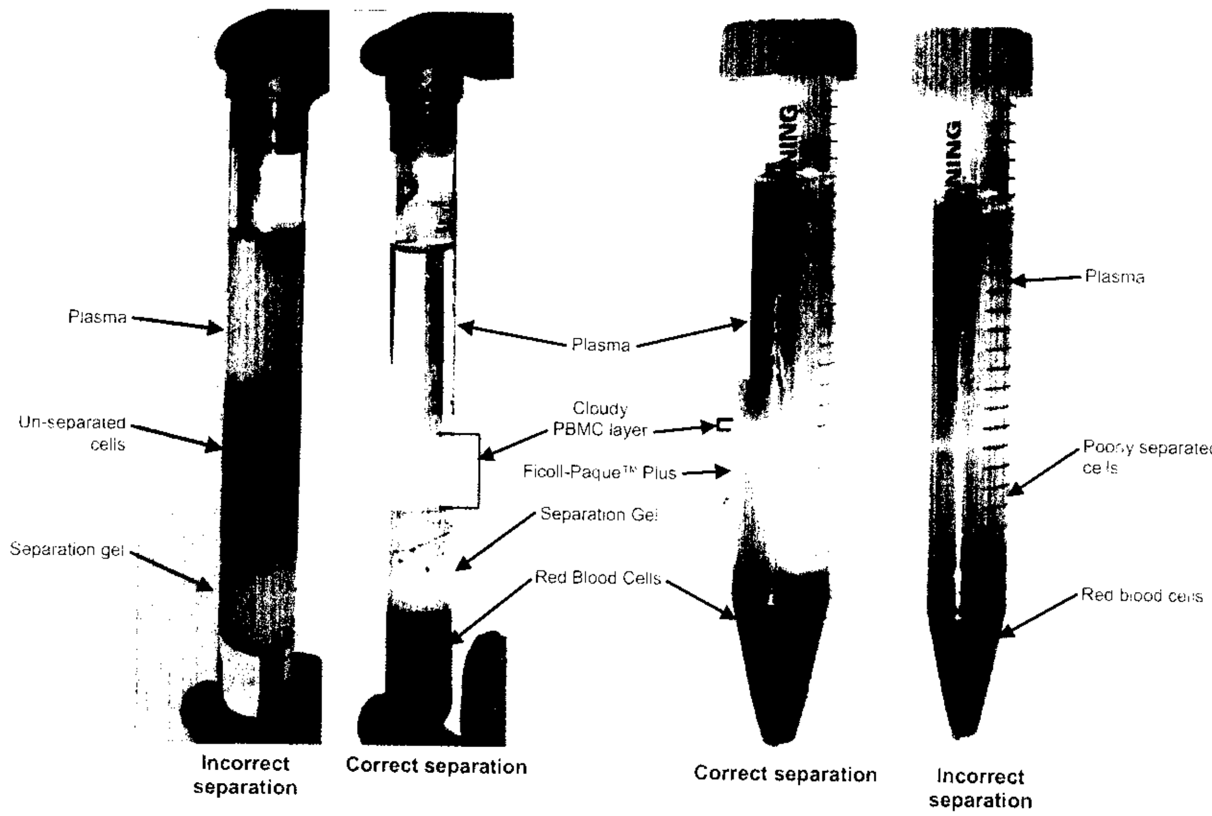


Figure 6. Incorrectly and correctly separated CPT method

Figure 7. Incorrectly and correctly separated Ficoll-Paque method

If correct separation has not been achieved, it may be caused by the use of the wrong centrifugation speed. If the tubes were centrifuged in RPM instead of RCF (as required), assure appropriate RCF to RPM conversion and centrifuge again.

Where separation is not achieved the following should be checked:

- Were the appropriate blood collection tubes used and were the tubes stored appropriately before use?
- Were the blood samples stored at room temperature (18-25°C)?
- Were the tubes inverted to mix the samples thoroughly with anticoagulant?
- Was the Ficoll gradient set up according to the method described in this section?
- Was the appropriate centrifugation speed used and was the centrifuge brake turned off?
- Was the blood sample processed on the day of blood collection (within 8 hours)?

Package Insert Instructions For Use

3. Collect the white, cloudy band of PBMCs using a pipette and transfer to a 15mL conical centrifuge tube. Bring the volume to 10mL with cell culture medium.

Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs.

Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs.

Training Notes and Visual Aids

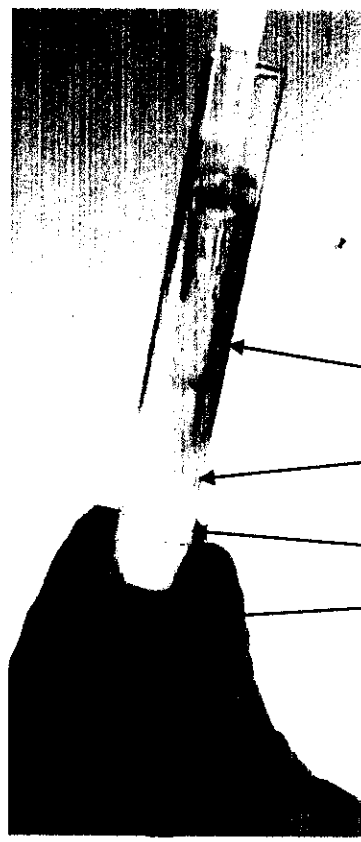


Figure 8. Removal of the cloudy PBMC layer from a CPT with a 1mL pipette

Insert a pipette tip down through the plasma to the cloudy layer and aspirate the liquid into the pipette tip (Figure 8). Transfer to a 15 mL conical centrifuge tube.

The centrifuge tubes require a conical bottom (see example in Figure 10) so that a pellet is formed when centrifuged in the next step. Falcon (blue top) and Corning (orange top) centrifuge tubes are known to perform well.

Notes:

The use of a 1mL pipette tip or other wide bore tip is recommended to reduce any possible damage to the cells.

Ensure that all of the cloudy PBMC layer is collected. It is better to take some of the plasma layer than to leave any of the PBMCs in the blood collection tube. However, if using CPTs avoid transferring any of the separation gel, which can block the pipette tip. If this happens transfer the cells already in the tip into a centrifuge tube and then use a new pipette tip to transfer the remaining PBMCs.

A variety of media can be used for washing the cells during steps 3-5; both AIM-V and RPMI 1640 have been used successfully and are recommended.

Package Insert Instructions For Use

4. Centrifuge at 600 RCF (g) for 7 minutes. Pour off the supernatant and resuspend the pellet in 1mL medium.
5. Bring the volume to 10mL with fresh medium and centrifuge at 350 RCF (g) for 7 minutes.
6. Pour off the supernatant and resuspend the pellet in 0.7mL cell culture medium.

The serum-free medium AIM-V has been used successfully and is strongly recommended

Training Notes and Visual Aids

Figure 9 Fill the 15mL tube up to 10mL with culture medium.

Figure 10: Resuspend the cell pellet in 0.7mL of AIM-V

Important notes for washing PBMCs

- Cell culture media for the washing steps should be pre-warmed to 37°C before contact with PBMCs
- It is strongly recommended that cells are resuspended in AIM-V for the overnight incubation (Note: Although RPMI 1640 is suitable for washing PBMCs it should not be used for overnight incubation.)
- Cells should be mixed by either gentle swirling of the tube by hand, or by gently agitating the suspension by pipetting the suspension up and down several times. This will ensure that the cells are evenly distributed

Notes:

To avoid problems with contaminated media, dispense 500mL bottles of AIM-V or RPMI 1640 aseptically into smaller aliquots e.g. 10x 50mL aliquots. For instance, this allows a 50mL aliquot of AIM-V to be used per 24 tests (1x T-SPOT.TB kit) when RPMI is used for cell washing.

If preferred the set-up of the plate can be started during the centrifugation steps (see page 14 Plate Set Up and Incubation)

After centrifugation, check for a cell pellet at the bottom of the tube. If a pellet hasn't formed check that the correct centrifugation speed was used and repeat the step.

Use a 1mL pipette to resuspend the cells. Expel 1mL of media with force, with the tip aimed at the wall near the pellet. This will create a vortex which should break up the pellet. If this fails to break up the pellet, media can be aspirated and expelled using the same pipette tip.

If too few cells are suspected, resuspend the pellet in 0.5mL AIM-V.

CELL COUNTING AND DILUTION

Package Insert Instructions For Use

CELL COUNTING AND DILUTION

T-SPOT.*TB* requires 250,000± 50,000 PBMCs per well. A total of four wells are required for each patient sample; thus 1 X 10^6 PBMCs are required per patient. The number of *M. tuberculosis* T cells in the specimen is normalized to a fixed number of PBMCs.

1. Perform a PBMC count. Cells can be counted by a variety of methods, including manual counting using Trypan Blue (or other appropriate stain) and a hemocytometer, or using an automated hematology analyzer.

2. Briefly, for manual counting with a Neubauer hemocytometer using Trypan Blue, add 10µL of the final cell suspension to 40µL 0.4%(w/v) Trypan Blue solution. Place an appropriate aliquot onto the hemocytometer and count the cells in the grid. For other types of hemocytometer and for automated devices, follow the manufacturers' instructions.

Training Notes and Visual Aids

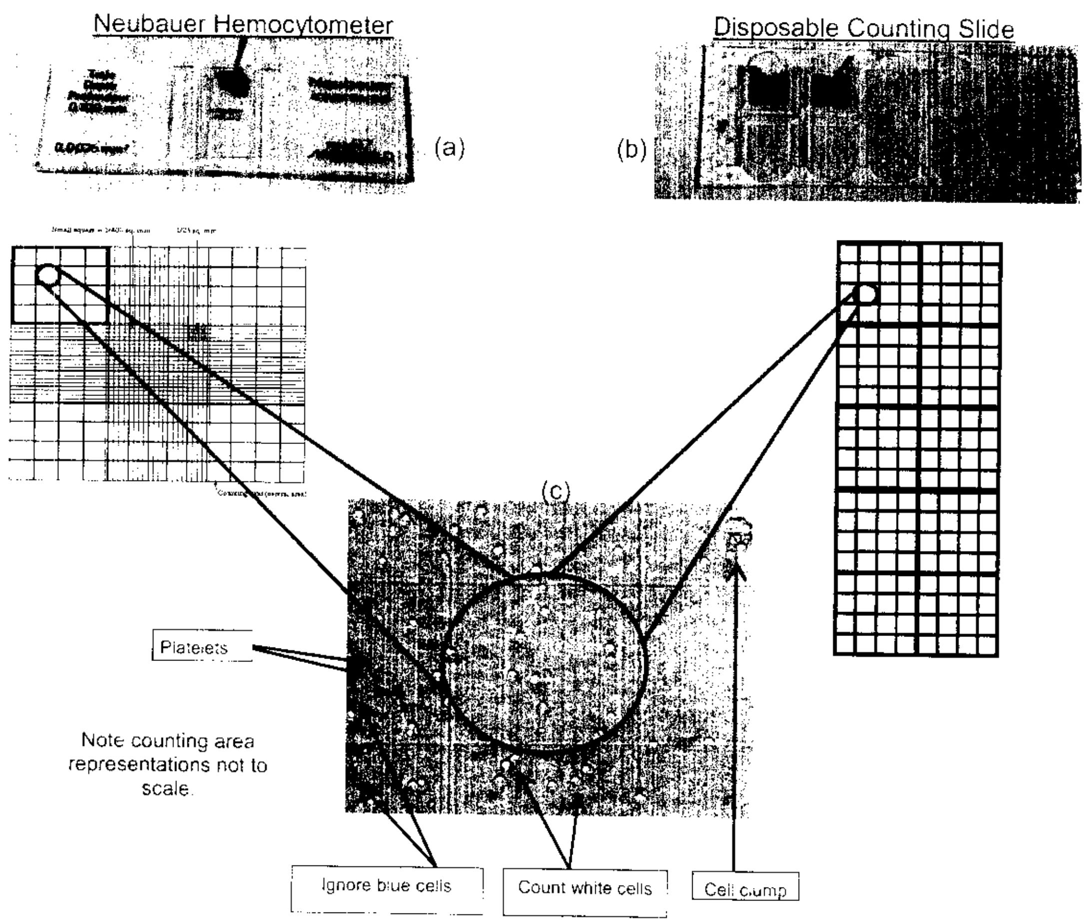


Figure 11: Sample chamber and counting areas of (a) Neubauer hemocytometer and (b) Disposable Counting Slide. Cells stained with Trypan Blue as seen under a microscope using the x10 objective (c)

Cell concentration can be determined by counting the number of cells within a defined volume. A Neubauer hemocytometer contains two chambers, which are divided into nine major squares (*Figure 11 (a)*). These have a volume of $0.1mm^3$ or 1 x 10^{-4}mL each. Disposable counting slides are an optically clear plastic slide consisting of 10 separate counting chambers with integral coverslip. Each of these counting areas consists of ten major squares that have a volume of $0.1mm^3$ or 1 x 10^{-4}mL each (*Figure 11 (b)*).

Count the cells in one major square (highlighted in red in *Figure 11*) of the counting grid using the low-power (x10) objective to count the number of cells within the appropriately defined area. When counting the cells that touch the perimeter lines, count only those cells that touch the left and upper outside lines and disregard those that touch the right and lower lines. This is to avoid counting the same cells twice. An example of cells that have been stained with Trypan blue is shown in *Figu3e 11 (c)*.

Package Insert Instructions For

3. Calculate the concentration of PBMCs present in the stock cell suspension.

4. Prepare 500µL of the final cell suspension at a concentration of 2.5×10^5 cells/100µL (giving 1.25×10^6 PBMCs in total).

Notes:

Care should be taken to ensure that the cell suspension is well mixed immediately prior to removal of aliquots for dilution or for counting Cells can settle towards the bottom of the tube leading to a misinterpretation of the true cell number. Mixing should be done by either gentle swirling of the tube by hand, or by gently agitating the suspension by pipetting the suspension up and down several times.

Ensure the calculation is correct for the cell counting system used as the use of either insufficient or excess cells may lead to an incorrect interpretation of the result.

Ensure cells are thoroughly mixed, by gently agitating the suspension by pipetting the suspension up and down several times, before removing an aliquot for dilution. PBMC numbers between 200,000 and 300,000 per well have been shown to give consistent T-SPOT.TB results.

Training Notes and Visual Aids

Manual Counting example

Calculate the concentration of PBMCs in the stock cell suspension by using the following equation:

$$\text{Volume of cell suspension required to prepare cell dilution (mL)} = \frac{25}{\text{Number of cells counted}}$$

This can only be used when the dilution factor is 5 and the volume of the area counted is 0.1µL.

Example:

For a cell count of 125:

$$\frac{25}{125} = \textbf{200µL} \text{ cell suspension}$$

Bring this volume of cell suspension to the required volume of **500µL** by adding **300µL** AIM-V medium or other serum-free cell culture medium. This gives a final solution of 250,000 cells/100µL for use in the assay.

Automated Counting

A hematology analyzer (automated cell counter) can be used to calculate the number of White Blood Cells (WBC) per mL (cells/mL) in the stock cell suspension in order to determine cell concentration.

The following equation should be used when using a hematology analyzer:

$$\text{Volume of cell suspension required to prepare cell dilution (mL)} = \frac{1.25}{N}$$

where N is the initial cell concentration represented as millions of cells/mL.

Example:

For a cell concentration of 10 million cells/mL:

$$\frac{1.25}{10} = \mathbf{125\mu L}\ \text{cell suspension}$$

Bring this volume of cell suspension to the required volume of **500µL** by adding **375µL** AIM-V medium. This gives a final solution of 250,000 cells/100µL for use in the assay.

Notes:
When using a hematology analyzer, check the length of the probe on the analyzer. It may not be long enough to reach the cell suspension in the centrifuge tube. In this case, transfer some of the liquid into a small vial (ideally with a capacity of 2 – 5mL) for sampling on the analyzer. Ensure that the analyzer is programmed to count white blood cells only.

Check the volume of sample that the hematology analyzer requires. Analyzers typically use between 100µL and 400µL. 4 x 100µL of cell suspension will be added to the microtiter plate wells when running the assay so if the analyzer uses 400µL, you will need at least 900µL of cell suspension allowing for some dead volume. In this case, the final volume of the cell suspension should be 1mL rather than 0.7mL.

Precautions:
Where insufficient cells are obtained to perform the assay the following should be checked:

- Was the appropriate volume of blood drawn from the patient?
- Was the counting chamber used correctly?
- Were the correct calculations used when calculating number of cells/mL and dilutions?
- Were the cells thoroughly mixed prior to removing an aliquot for counting?

PLATE SET UP AND INCUBATION

Oxford Immunotec

Harnessing the power of T cell measurement

PLATE SET UP AND INCUBATION

T-SPOT.*TB* requires four wells to be used for each patient sample. A Nil Control and a Positive Control should be run with each individual sample. It is recommended that the samples be arranged vertically on the plate as illustrated below.

- Nil Control
- Panel A (ESAT-6)
- Panel B (CFP10)
- Positive Control

Each 96-well plate can process up to 24 patient samples. Use the numbers of plates required for the numbers of samples that you wish to process. The 8-well strip version of T-SPOT.*TB* (T-SPOT.*TB 8*) provides the additional flexibility of 12 x 8-well strips. Each strip will process 2 samples. If you have purchased this version, use only the numbers of strips that you require.

T-SPOT.*TB* is an assay that measures T cell function; no standard curves are required. Therefore each patient will only require 4 wells to be used for each sample. The recommended plate layout for 24 samples is shown below:

Row	1	2	3	4	5	6	7	8	9	10	11	12
A	1N		5N		9N		13N		17N		21N	
B	1A		5A		9A		13A		17A		21A	
C	1B		5B		9B		13B		17B		21B	
D	1M		5M		9M		13M		17M		21M	
E		4N		8N		12N		16N		20N		24N
F		4A		8A		12A		16A		20A		24A
G		4B		8B		12B		16B		20B		24B
H		4M		8M		12M		16M		20M		24M

Key: N=nil control, A=Panel A, B=Panel B, M=Mitogen Positive Control)

1. Remove the pre-coated 8-well strips from the packaging, clip into a plate frame and allow to equilibrate to room temperature. Remove the required number of strips only, reseal any remaining unused strips and the desiccant pouch in the outer foil packaging and return to storage at 2-8°C. Alternatively, if the 96-well version is being used, remove the plate from the pouch and allow it to equilibrate to room temperature.

2. Add in the Panels and the Controls;

Add 50µL AIM-V cell culture medium to each Nil Control well
Add 50µL Panel A solution to each well required
Add 50µL Panel B solution to each well required
Add 50µL Positive Control solution to each cell functionality control well

Do not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause artifacts in the wells.

Training Notes and Visual Aids

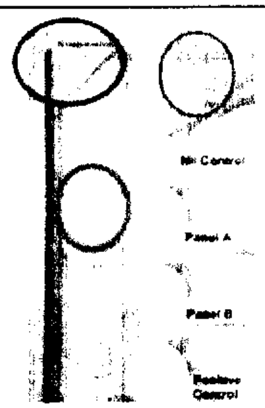


Figure 13. 4 wells used for one patient A1 to D1. Nil Control, Panel A, Panel B and Positive control respectively

The plate orientation should be as shown (Figure 13). Position the A1 well at the top left hand corner. This corner is clearly distinguishable by the flattened section shown in the blue circle. The column numbers are positioned along the top edge of the plate, shown in the red circle. The row letters are shown in the green circle down the left hand side of the plate.

Figure 13 shows 4 wells used for one patient sample positioned in the top four wells on the first strip of an 8 well strip plate (TB.300). The suggested orientation of wells to be used are shown e.g. A1 Nil Control, B1 Panel A, C1 Panel B and D1 Positive Control. Patient wells should be arranged in this order to avoid cross contamination when adding the positive control and patient cells.

Package Insert Instructions For Use

3. To each of the 4 wells to be used for a patient sample, add 100µL of the patient's final cell suspension (containing 250,000 cells). Use a new tip for the addition of each individual patient's cells to avoid cross-contamination between wells. **Note: Take care not to contaminate adjacent wells, by passing liquid from one well to another if pipette tips are reused for multiple wells.**

Training Notes and Visual Aids

Clip the strips to be used into an empty plate frame fitted with a bottom plate and lid. Figure 14 (left), shows a breakdown of the 8 well strip plate (TB.300) containing 2 strips: Plate frame (A), Lid (B), bottom plate (C), and the 2x 8 well strips (D). Figure 14 (right), shows the complete frame containing 2 strips (16 wells) sufficient for 4 patient samples. The frames, bottom plate and lids can be retained and reused.

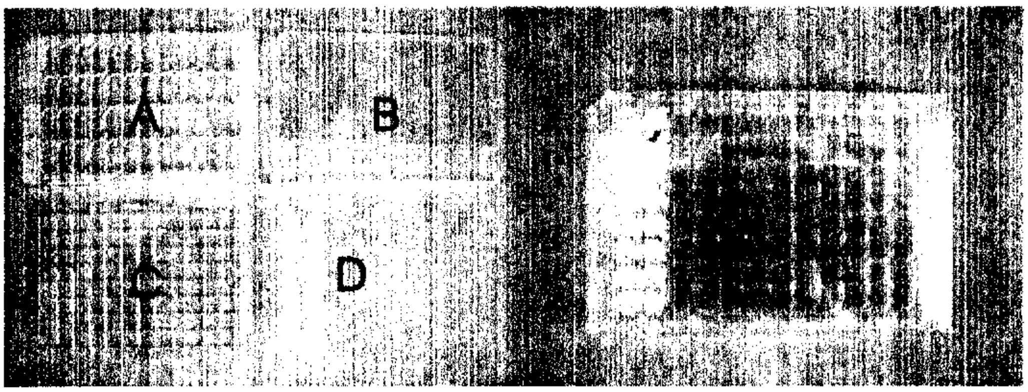


Figure 14: Left: Breakdown of the 8 well strip plate (TB.300) Right: Final assembly with 2 strips. Enough wells for 4 patient samples (e.g. 16 wells)

Plate, Panel A, Panel B, Positive Control (PHA) should all be at room temperature (18-25°C). AIM-V must be pre-warmed to 37°C.

Prior to pipetting, gently swirl cell suspension or gently pipette up and down to ensure cells are evenly distributed.

Do Not allow the pipette tip to touch the membrane. Indentations in the membrane caused by pipette tips may cause damage to the wells. If necessary, rest the tip on the side of the well.

Change tip when changing from one reagent to the next.

Package Insert Instructions For Use

4. Incubate the plate with the lid on in a humidified incubator at 37°C ± 1°C with 5% CO^2 supply for 16-20 hours. Avoid disturbing the plate once in the incubator. Do not stack plates as this may lead to uneven temperature distribution and ventilation.

Training Notes and Visual Aids

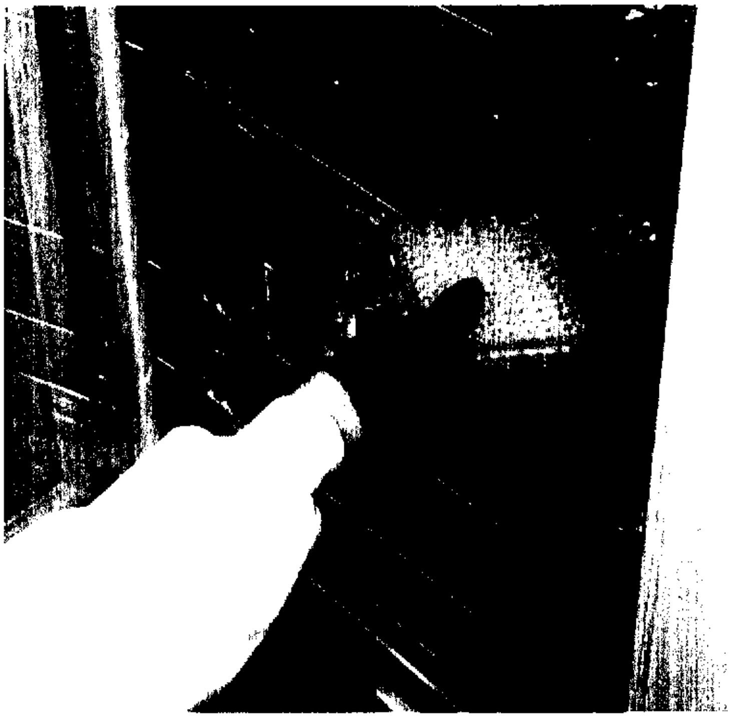


Figure 15: Place plate into incubator for 16-20 hours

Incubate plate in a humidified incubator at 37°C ± 1°C with 5% CO^2 supply (Figure 15). Check the water dish for sufficient water to ensure that a humid atmosphere is achieved.

SPOT DEVELOPMENT AND COUNTING

SPOT DEVELOPMENT AND COUNTING

1. Remove the plate from the incubator and discard the cell culture medium by flicking the contents into an appropriate container. ***Note:*** *At this point remove the Substrate Solution from the kit and allow to equilibrate to room temperature for 1 hour.*

2. Add 200µL Phosphate Buffered Saline (PBS) solution to each well. **Do not use PBS containing Tween™ or other detergents, as this causes high background counts.**

3. Discard the PBS solution. Repeat the well washing an additional 3 times with fresh PBS solution for each wash.

Note: For washing, an automatic plate washer or an 8 channel or stepper pipette to manually wash plates may be used. Discard PBS into a suitable container after each wash. Do not use pipettes to remove the PBS as this risks damaging the membrane. If using a plate washer, ensure the manifold is adjusted so that the tips do not touch the membrane. After the final wash, tap the plate on lint-free towel to ensure all PBS is removed – any

Training Notes and Visual Aids

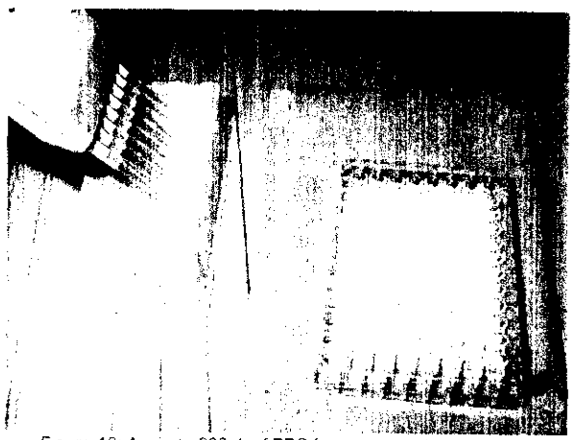


Figure 16: Aspirate 200µL of PBS from a reagent tray

Note:

For washing, an 8 channel or stepper pipette and a plastic reservoir to hold PBS may be used shown in Figure 16

Notes:

With force, dispense 200µL in to each well to wash thoroughly (Figure 17)

If using a plate washer, ensure the manifold is adjusted so that the tips do not touch the membrane. After the final wash, tap the plate on lint-free towel to ensure all PBS is removed – any excess left will further dilute the Conjugate Reagent.

When washing the plate, ensure that all the wells are full in between washes

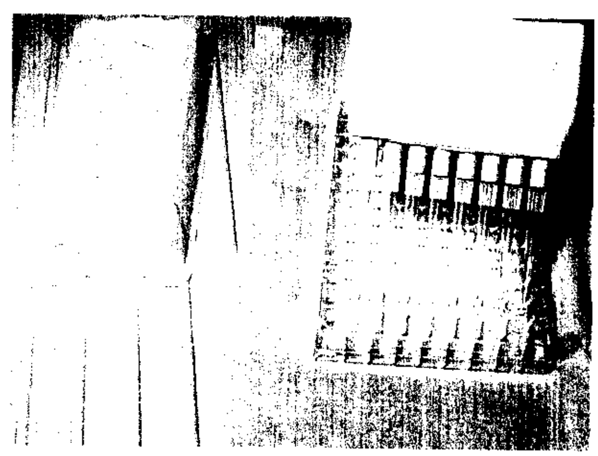


Figure 17: Dispense 200µL of PBS into each well

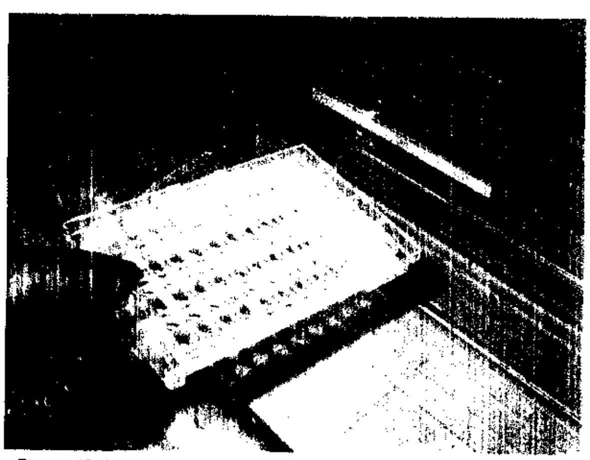


Figure 18: Invert plate into a container

Note:

Do not use pipettes to remove the PBS as this risks damaging the membrane.

Discard PBS by inverting the plate over a suitable container after each wash (Figure 18).

Package Insert Instructions For Use

4. If not already prepared during the reagent preparation step, dilute concentrated Conjugate Reagent 200X in PBS to create the working strength solution.

5. Add 50µL working strength Conjugate Reagent solution to each well and incubate at 2-8°C for 1 hour.

6. Discard the conjugate and perform the four PBS washes as described in steps 2 and 3 above.

7. Add 50µL Substrate Solution to each well and incubate at room temperature for 7 minuets.

8. Wash the plate thoroughly with distilled or deionised water to stop the detection reaction.

9. Allow the plate to dry in a well ventilated area or in an oven at up to 37°C. Spots become more visible as the plate dries; therefore ensure that the plate is thoroughly dry before reading. Allow 4 hours drying time at 37°C or at least 16 hours at room temperature.

Tips:

In order to load all wells quickly, a multi-channel pipette and a plastic reservoir is recommended.

Ensure all residual PBS is removed prior to adding the substrate solution.

It is recommended that an aliquot of Substrate Solution be removed from the reagent bottle to avoid risk of contamination.

Training Notes and Visual Aids

Figure 19: Perform Conjugate dilution (1:200)

Example Dilution:

Each patient sample will have 4 wells. 50µL diluted Conjugate Reagent will be added to each well. Thus, for one strip (2 samples, 8 wells), prepare 500µL of working strength solution by adding 2.5µL of concentrated Conjugate Reagent (use a 1-20 µL pipette) to 497 5µL PBS. Mix by inverting 5-6 times.

For one 96-well plate (24 wells) prepare 5mL of working strength solution by adding 25µL of concentrated Conjugate Reagent to 4975µL PBS.

Note: Twice as much Conjugate Reagent than is required is provided with each kit. Care should be taken to limit the amount of excess solution prepared (for wastage) to avoid running out of conjugate.

Care should be taken to ensure that the Conjugate Reagent is added to every well as the solution is clear and uncolored.

Note:

Use of an 8 channel or stepper pipette is recommended for pipetting the Substrate (Figure 20).

The substrate solution should be used at room temperature and is supplied ready to use.

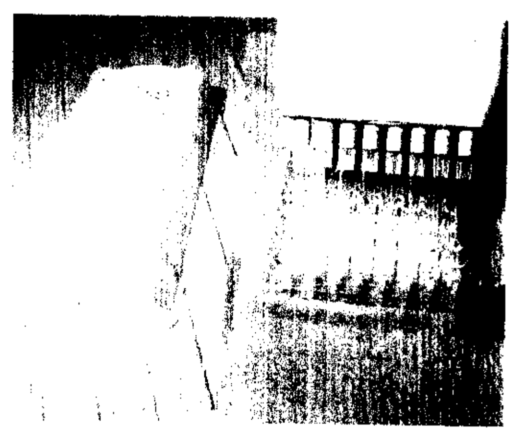


Figure 20: Add substrate and incubate at *18-25°C* for 7 mins

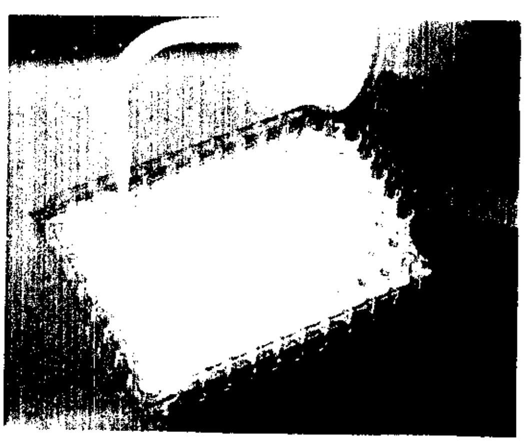


Figure 21: Wash wells with distilled or deionised water

After washing the plate with distilled or deionised water (Figure 21), tap out any residual liquid onto absorbent paper. As the wells dry, the background decreases and the spots become more sharply defined and thereby, easier to count. Ensure the plate is thoroughly dry before reading.

Package Insert Instructions For Use

10. Count and record the number of distinct, dark blue spots on the membrane of each well. Apply the Results Interpretation and Assay Criteria (see below) to determine whether a patient sample is 'Positive' or 'Negative'. **The spots produced as a result of antigen-stimulation should appear as large, round and dark spots. Often a gradient effect can be observed with a darker centre and a more diffuse periphery. Non specific artifacts that can occur are smaller, less intense and irregular in shape.**

Once developed, the completed assay plates remain stable and they do not, therefore, need to be read immediately. The plates may be archived for retrospective quality control or re-examination for up to 12 months if kept in a dry, dark environment at room temperature.

Training Notes and Visual Aids

Example Results

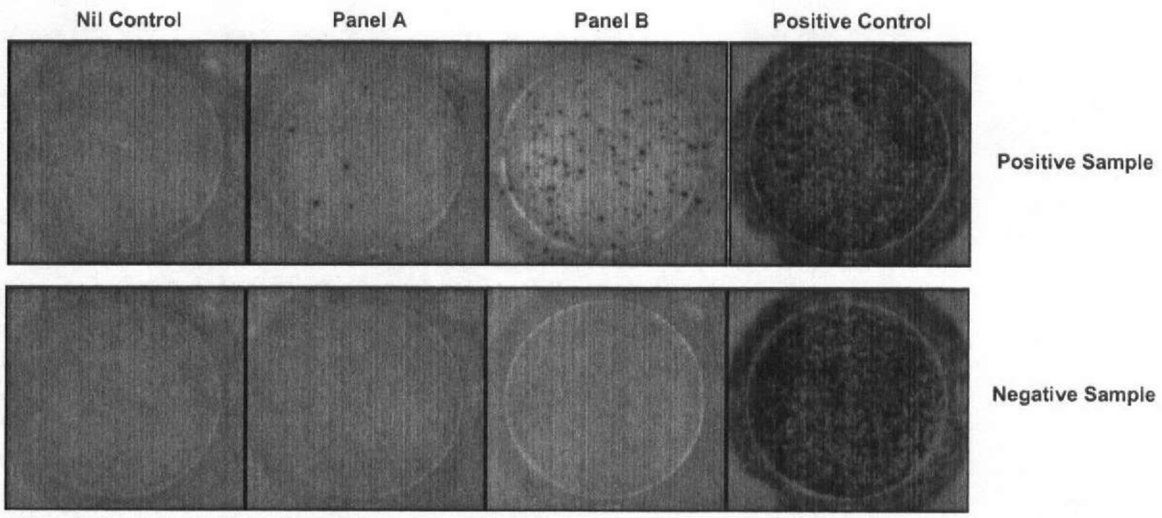


Figure 22: Two typical T-SPOT.*TB* results. Top = Positive. Bottom = Negative.

Spots can be counted directly from the well using a magnifying glass, a stereomicroscope, or from a digital image captured from a low powered microscope.

QUALITY CONTROL

QUALITY CONTROL

A typical result would be expected to have few or no spots in the Nil Control and 20 or more spots in the Positive Control

High numbers of spots in the Nil Control may occur. In addition, high background staining in one or more wells may occur which makes counting of spots difficult. If high background staining occurs such that discrimination of the spots from the background is hindered, the results should be considered invalid These results are usually due to operator issues such as: suboptimal plate washing, medium contamination or inappropriate specimen handling and PBMC separation methods. It is, however, possible that the state of health of the patient may produce this effect in a small number of cases

A Nil Control spot count in excess of 10 spots should be considered as 'Invalid'.

Typically, the cell functionality Positive Control spot count should be ≥ 20 or show saturation (too many spots to count). A small proportion of patients may have T cells which show only a limited response to PHA[3] Where the Positive Control spot count is < 20 spots, it should be considered as 'Invalid', unless either Panel A or Panel B are 'Positive' or 'Borderline (equivocal)' as described in the Results Interpretation and Assay Criteria (see below), in which case the result is valid

In the case of Invalid results, these should be reported as "Invalid" and it is recommended to collect a further sample and re-test the individual

Training Notes and Visual Aids

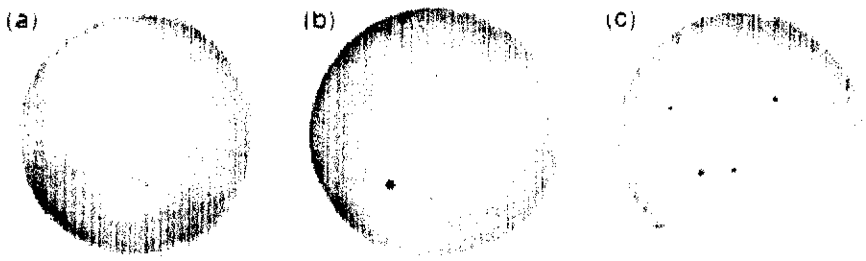


Figure 23 Typical images from Nil control wells. (a) shows a well with 0 spots (b) shows a well with 1 spot and (c) shows a invalid Nil control well of > 10 spots

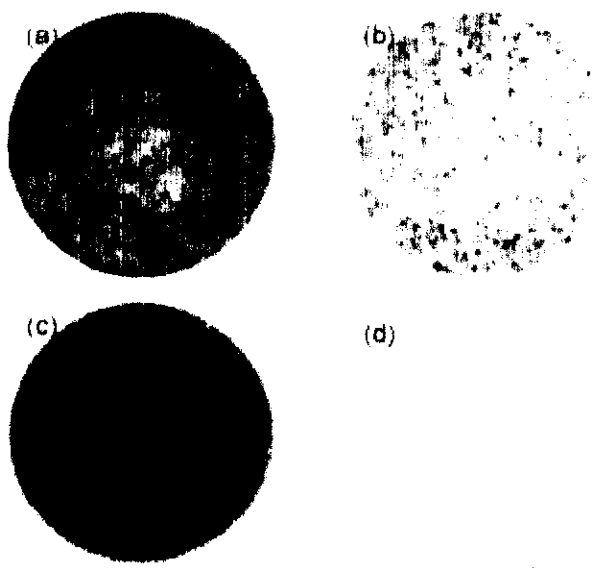


Figure 24 Typical images from Positive control wells (a) (b) and (c) all shown acceptable responses for patient samples. Figure (d) shows an invalid Positive control well due to < 20 spots

RESULT INTERPRETATION

RESULTS INTERPRETATION AND ASSAY CRITERIA

Refer to the Quality Control section before applying the following criteria.

NOTE: Diagnosing or excluding tuberculosis disease, and assessing the probability of LTBI, requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting T-SPOT.*TB* Refer to the most recent CDC guidance (http://www.cdc.gov/nchstp/tb) for detailed recommendations about diagnosing TB infection (including disease) and selecting persons for testing.

Results for T-SPOT.*TB* are interpreted by subtracting the spot count in the Nil control well from the spot count in each of the Panels, according to the following algorithm:

The test result is Positive if (Panel A-Nil) and/or (Panel B-Nil) ≥ 8 spots

The test result is Negative if both (Panel A-Nil) and (Panel B-Nil) ≤ 4 spots. This include results less than zero.

Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5,6 or 7 spots should be considered Borderline (equivocal) and retesting by collecting another patient specimen is recommended.

If the result is still Borderline (equivocal) on retesting with another specimen, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

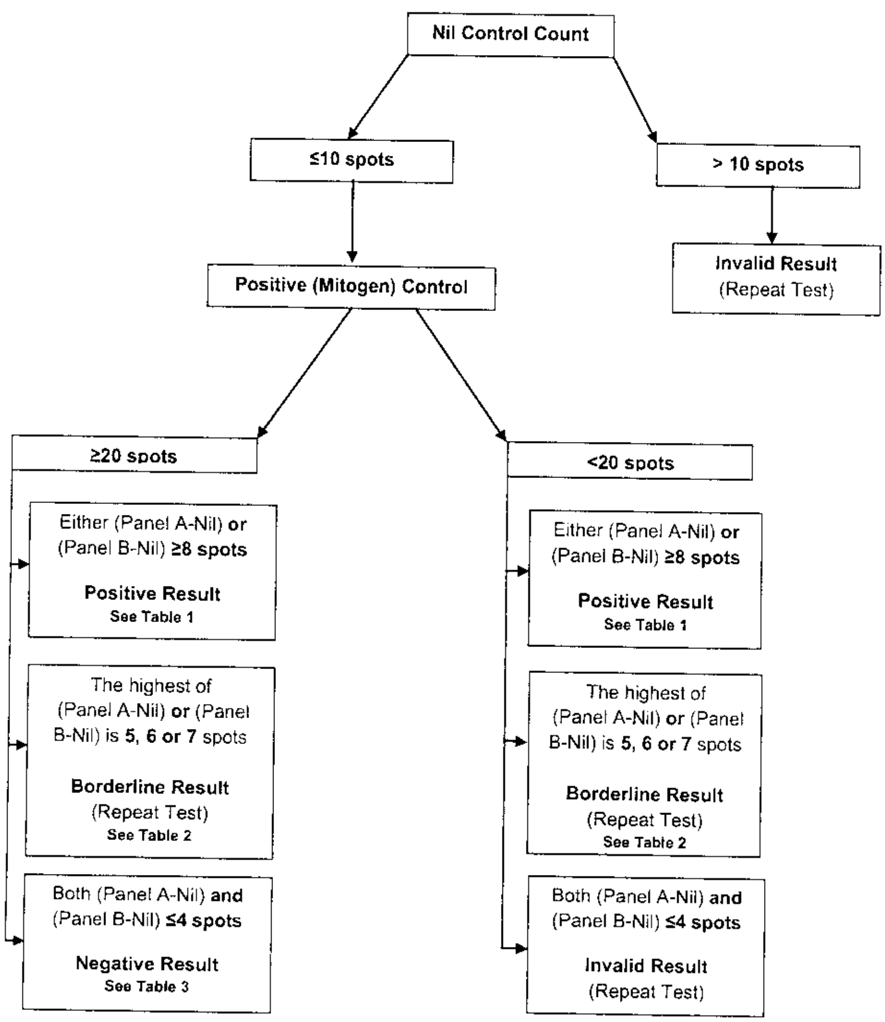


Table 1: Positive Interpretation: Either (Panel A-Nil) or (Panel B-Nil) ≥8 spots

Nil Control Well Count	Either Panel A **or** Panel B has the following number of spots†	Result Interpretation
0	≥8	**Positive**
1	≥9	**Positive**
2	≥10	**Positive**
3	≥11	**Positive**
4	≥12	**Positive**
5	≥13	**Positive**
6	≥14	**Positive**
7	≥15	**Positive**
8	≥16	**Positive**
9	≥17	**Positive**
10	≥18	**Positive**
>10 spots	n/a	**Invalid**

†Note: The Panel with the highest number of spots is used for the calculation.

Table 2: Borderline (equivocal) Interpretation: The highest of (Panel A-Nil) or (Panel B-Nil) is 5, 6 or 7 spots

Nil Control Well Count	The highest of Panel A **or** Panel B has the following number of spots	Result Interpretation
0	5, 6, or 7	**Borderline (equivocal)***
1	6, 7, or 8	**Borderline (equivocal)***
2	7, 8, or 9	**Borderline (equivocal)***
3	8, 9, or 10	**Borderline (equivocal)***
4	9, 10, or 11	**Borderline (equivocal)***
5	10, 11, or 12	**Borderline (equivocal)***
6	11, 12, or 13	**Borderline (equivocal)***
7	12, 13, or 14	**Borderline (equivocal)***
8	13, 14, or 15	**Borderline (equivocal)***
9	14, 15, or 16	**Borderline (equivocal)***
10	15, 16, or 17	**Borderline (equivocal)***
>10 spots	n/a	**Invalid****

Table 3: Negative Interpretation: Both (Panel A-Nil) and (Panel B-Nil) ≤4 spots

Nil Control Well Count	Both Panel A and Panel B has the following number of spots	Result Interpretation
0	≤4	**Negative**
1	≤5	**Negative**
2	≤6	**Negative**
3	≤7	**Negative**
4	≤8	**Negative**
5	≤9	**Negative**
6	≤10	**Negative**
7	≤11	**Negative**
8	≤12	**Negative**
9	≤13	**Negative**
10	≤14	**Negative**
>10 spots	n/a	**Invalid****

*Results where the highest of the Panel A or Panel B spot count is such that the (Panel minus Nil) spot count is 5,6 or 7 spots should be considered Borderline (equivocal) and retesting by collecting another patient specimen is recommended.
** In the case of Invalid results, these should be reported as "Invalid" and it is recommended to collect another sample and re-test the individual.

TROUBLESHOOTING

This assay should be performed using the principles of Good Laboratory Practice and by strictly adhering to these Instructions for Use.

Borderline (equivocal) Results

Borderline (equivocal) results are those where the maximum of the two (Panel minus Nil) spot count results are within ±1 spots from the ROC-determined assay cutoff of ≥6 spots. Borderline (equivocal) results, although valid, are less reliable than results where the spot count is further from the cut-off. Retesting of the patient, using a new sample, is therefore recommended. If the result is still Borderline (equivocal) on retesting, then other diagnostic tests and/or epidemiologic information should be used to help determine TB infection status of the patient.

Invalid Results

Invalid results are uncommon and may be related to the immune status of the individual being tested[12]. They may also be related to a number of technical factors, potentially resulting in "high background", "low mitogen", and "high nil" results such as:

- Use of inappropriate blood collection tubes
- Storage of blood greater than 8 hours prior to processing
- Storage of blood outside the recommended temperature range (18-25°C prior to processing)
- Contamination of the cell culture media
- Incomplete plate washing

Repeating the test using a new patient sample is recommended for invalid results. Technical documents are available covering key troubleshooting points. These are available by contacting Oxford Immunotec.

For Technical Support in the United States contact: 1 – 877 – 20-TSPOT (87768).

SPOT INTERPRETATION — ADDITIONAL INFORMATION

Panel A and B Antigens

The number of spots in the antigen wells can vary from zero to several hundred. High spot numbers will be difficult and time-consuming to count so may be recorded as >20 spots. Examples of typical wells are shown in Figure 26.

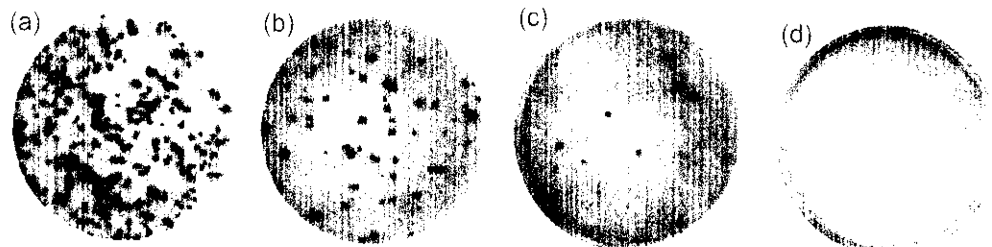


Figure 26: Typical images from Panel A and B antigen wells. (a) Positive sample (>20 spots); (b) Positive sample (>20 spots); (c) Positive sample (8 spots) and (d) Negative sample (0 spots)

Example patient interpretations:

	Well		Spot count	Valid?
Patient 1.	Nil Control	=	2	✓
	Panel A	=	11	✓
	Panel B	=	1	✓
	Positive Control	=	>20	✓

Panel A value subtract the Nil control value = 9 (9 ≥8) therefore. **Result = <u>Positive</u>**

	Well		Spot count	Valid?
Patient 2.	Nil Control	=	1	✓
	Panel A	=	0	✓
	Panel B	=	2	✓
	Positive Control	=	>20	✓

Panel B value subtract the Nil control value = 1 (1 ≤4) therefore: **Result = <u>Negative</u>**

	Well		Spot count	Valid?
Patient 3.	Nil Control	=	11	X
	Panel A	=	13	✓
	Panel B	=	12	✓
	Positive Control	=	>20	✓

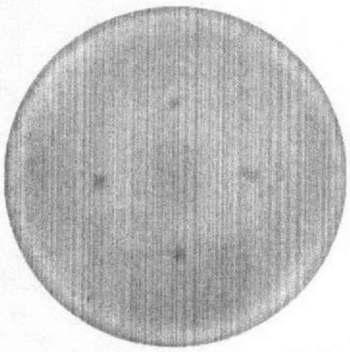
Nil control value >10 therefore: **Result = <u>Invalid (Repeat Test)</u>**

	Well		Spot count	Valid?
Patient 4.	Nil Control	=	0	✓
	Panel A	=	0	✓
	Panel B	=	7	✓
	Positive Control	=	<20	X

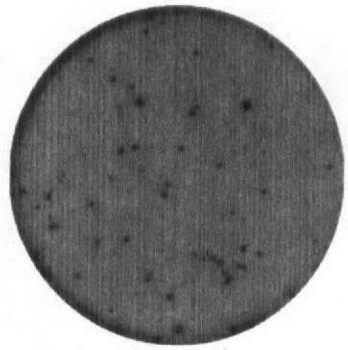
Positive control value <20 however, Panel B value subtract the Nil control value = 7 therefore **Result = <u>Borderline (equivocal) (Repeat Test)</u>**

SPOT INTERPRETATION — TROUBLESHOOTING

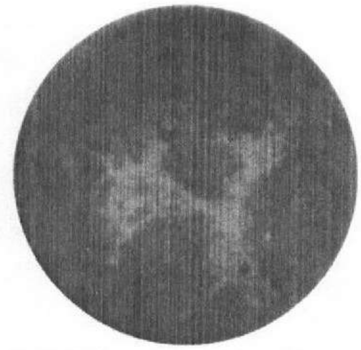
If wells contain debris or have a high background care should be taken when counting spots. See the examples below:



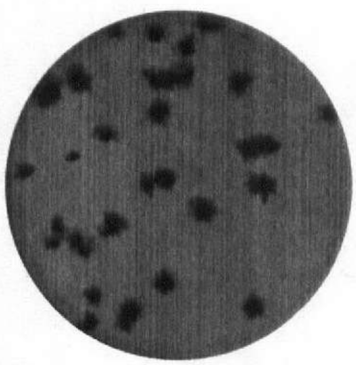
Example 1: 4 Mark Effect. These marks can be attributed to over excessive pressure on the back of the solid 96 well plate (TB.200) caused when tapping solutions out of the plate during the plate washing steps. [In this case the spot count = 0].



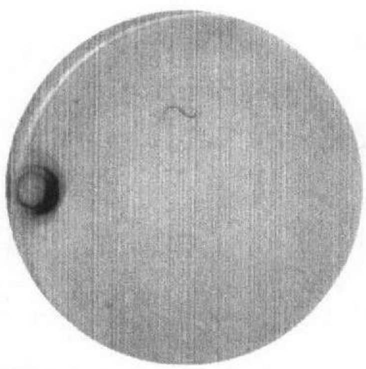
Example 2: High background in panel well. Although a high background is uncommon with T-SPOT.*TB*, spots can still be seen over background. [In this case the spot count = >20].



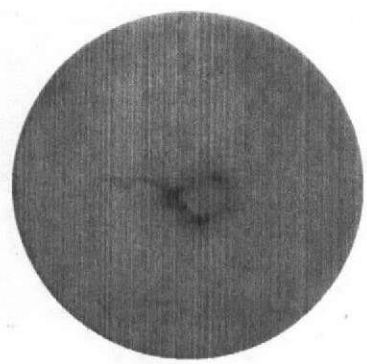
Example 3: Excessively high background can be produced, possibly due to: suboptimal plate washing, medium contamination or inappropriate specimen handling and PBMC separation. It is also possible that the state of health of the patient may produce this effect in a small number of cases. [In this case the result for this Nil control well was invalid]



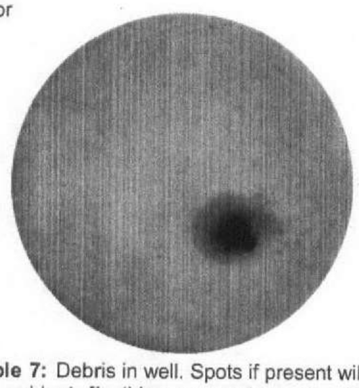
Example 4: Fungal contamination in the well. These growths are not round in shape. Rather they appear as not uniform and have fibrous edges. They are also much larger than ELISPOT spots. [In this case spot Count = 0]. Care should be taken not to allow a contamination to occur. Check incubator, medium and assay reagents for possible contamination.



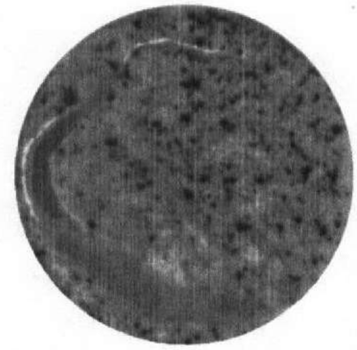
Example 5: Pipette tips can cause dark marks on the membrane. However spots will still be evident. [In this case the spot count = 0]. Care should be taken when pipetting reagents and cells to not touch the membrane with pipette tips.



Example 6: Debris in well may be visible e.g. a hair. However spots will still be evident. [In this case the spot count = 0]. Care should be taken when pipetting cells and reagents to not allow debris to fall into the assay wells.



Example 7: Debris in well. Spots if present will still be evident. [In this case spot count = 0]. Care should be taken when pipetting cells and reagents into the plates to not allow environmental debris to fall into the assay wells.



Example 8: Broken membrane. Spots are still evident in this positive control well. [In this case spot count = >20]. Care should be taken when pipetting into the wells to ensure that the membranes are not dam-

References

1. NCCLS. Performance of single cell immune response assays; approved guideline. NCCLS document I/LA26-A.
2. Meier T et al. Sensitivity of a new commercial enzyme-linked immunospot assay (T SPOT-TB) for diagnosis of tuberculosis in clinical practice. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2005; 24: 529-536.
3. Köller MD et al., Functional and molecular aspects of transient T cell unresponsiveness: role of selective Interleukin-2 deficiency. *Clin Exp Immunol.* 2003 May;132(2):225-31.

Oxford Immunotec, Inc.
2 Mount Royal Ave, Ste. 100
Marlborough, MA 01752 USA
Toll Free: 877-20 TSPOT
www.oxfordimmunotec.com

Oxford Immunotec, Ltd.
94C Milton Park, Abingdon
Oxfordshire, OX14 4RY, UK
Tel: +44 (0) 1235 442796

Oxford Immunotec

Harnessing the power of T cell measurement